Active TEM-1 β -lactamase mutants with random peptides inserted in three contiguous surface loops

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Abstract

Engineering of alternative binding sites on the surface of an enzyme while preserving the enzymatic activity would offer new opportunities for controlling the activity by binding of non-natural ligands. Loops and turns are the natural substructures in which binding sites might be engineered with this purpose. We have genetically inserted random peptide sequences into three relatively rigid and contiguous loops of the TEM-1 β -lactamase and assessed the tolerance to insertion by the percentage of active mutants. Our results indicate that tolerance to insertion could not be correlated to tolerance to mutagenesis. A turn between two β -strands bordering the active site was observed to be tolerant to random mutagenesis but not to insertions. Two rigid loops comprising rather well-conserved amino acid residues tolerated insertions, although with some constraints. Insertions between the N-terminal helix and the first β -strand generated active libraries if cysteine residues were included at both ends of the insert, suggesting the requirement for a stabilizing disulfide bridge. Random sequences were relatively well accommodated within the loop connecting the final β -strand to the C-terminal helix, particularly if the wild-type residue was retained at one of the loops' end. This suggests two strategies for increasing the percentage of active mutants in insertion libraries. The amino acid distribution in the engineered loops was analyzed and found to be less biased against hydrophobic residues than in natural medium-sized loops. The combination of these activity-selected libraries generated a huge library containing active hybrid enzymes with all three loops modified.

Keywords: β -lactamase; insertion tolerance; in vivo selection; loop engineering

The development of modified proteins with new binding specificities and affinities for defined targets is one of the major goals of protein engineering. Considerable efforts have been devoted to this field, motivated by well-documented limitations of antibodies for several analytical or biomedical applications. Binding sites have been created in many protein scaffolds either by the introduction of random mutations on the surface of contiguous

helices or strands, or by the randomization of the sequences of the loops connecting these secondary structures. In most cases, the selection of binding proteins from these combinatorial libraries mainly by phage display has led to the isolation of variants with high affinity for the target ligands (Skerra 2000; Mathonet and Fastrez 2004; Binz et al. 2005).

On the other hand, alternative binding sites have rarely been constructed in enzymes while paying attention to the preservation of activity. Insertions of defined epitopes within loops of alkaline phosphatase or β -galactosidase have led to the creation of molecular sensors in which the enzymatic activity could be modulated by low concentrations of antibodies specific for the inserted epitopes

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(Brennan et al. 1995; Feliu and Villaverde 1998; Ferrer-Mirallès et al. 2001). Libraries of phage displaying variants of TEM-1 β -lactamase in which random peptides were genetically inserted within the enzyme scaffold have also been created, and enzymes that had acquired an affinity for antibodies unrelated to the β -lactamase or for other proteins have been selected from these. They were frequently found to have their activity modulated by binding (Legendre et al. 1999, 2002). These successful experiments suggest that a regulation of enzymatic activity could be achieved by creation of binding sites within loops. Complex binding sites designed to bind small organic ligands could possibly be created if random peptide libraries could be simultaneously inserted in several contiguous regions of the three-dimensional structure. These enzymes would contain an allosteric site, which would share some characteristics with binding sites of camelid antibodies featuring only heavy chains and three loops for binding (Hamers-Casterman et al. 1993).

In this work, we analyze the possibility of extending up to three loops in the scaffold of the TEM1 β -lactamase using random peptide libraries. This enzyme, the best known member of the class A β -lactamases (Matagne and Frère 1995; Matagne et al. 1998), very efficiently hydrolyzes β -lactam antibiotics, particularly penicillins, and thus provides a mechanism of resistance against them. This allows *in vivo* selection of bacteria-producing active mutants. If the enzyme is displayed on phage, *in vitro* selection protocols can also be used to isolate active mutants from libraries (Soumillon et al. 1994; Vanwetswinkel et al. 2000). Being a rather stable enzyme (Vanhove et al. 1997), TEM-1 β -lactamase might be able to tolerate relatively large perturbations, such as the insertion of random peptide libraries within short loops. We examine its tolerance to the conversion of relatively rigid loops into presumably flexible medium size loops, which we will define as Ω -loops and compare the amino acid preferences in the active mutants with those seen in Ω -loops of natural proteins. We also report the results of combining libraries in which several loops were engineered simultaneously. Structural constraints for successful insertions are also evaluated.

Results

Sites of insertions

The criteria used to define the positions at which insertions were engineered into the β -lactamase scaffold were the solvent exposure and mobility of the loops and information on the tolerance of mutations and insertions. The position of solvent-exposed loops is known from the structure (Jelsch et al. 1993), and their mobility can be assessed from the crystallographic B-factors. Random

insertion mutagenesis experiments have given some indication of tolerance to insertion of dipeptides or pentapeptides in certain regions of the protein (Barany 1985; Zebala and Barany 1991; Hallet et al. 1997; Hayes et al. 1997). The sequence alignment of class A β -lactamases points to loops in which insertions or deletions have been naturally introduced. Tolerance to mutation has been thoroughly explored by saturation mutagenesis of the 263 codons of the enzyme (Huang et al. 1996). Since we wished to create a new binding site based on three contiguous loops on the β -lactamase surface, we chose to engineer loops that were in close proximity (Fig. 1). The annotations of the libraries created are given in Table 1.

The first loop engineered, loop 1 (L1), is located between strands β 3 and β 4. It consists of three residues, Ambler numbering Gly238, Glu240, and Arg241 (Ambler et al. 1991; residue 239 is missing in TEM β -lactamase), and is known to accept mutations despite its proximity to the active site (Huang et al. 1996). An insertion of one residue (position 239) is observed on sequence alignments of class A β -lactamases. We determined the tolerance of this loop to larger insertions by replacing the dipeptide Glu240-Arg241 by three, four, five, or six random residues (libraries fd-blaA-L₁^{3–6}). The next two loops are located in symmetrical $\alpha\beta$ and $\beta\alpha$ positions between helices and β -strands. Loop 2 (L2), between the N-terminal helix α 1 and strand β 1, tolerates very few

Figure 1. Structure of TEM-1 β -lactamase. The essential nucleophilic residue, Ser70, is shown in yellow; L₁, L₂, and L₃, the loops in which random peptides were inserted, are shown in green, blue, and orange, respectively.

Table 1. Diversity, percentage of active mutants selected on 20 $\mu\text{g/mL}$ ampicillin, and activity of libraries

Library	Mutations introduced	Library size	% Active mutants	Activity (k_{cat}) of selected libraries
fd-bla	Wild type	—	—	1500 sec^{-1}
fd-blaA-L ₁ ³	E ₂₄₀ R $\rightarrow$ (X) _{3–6}	8×10^5	<0.1%	NS
fd-blaA-L ₁ ⁴		6×10^6	<0.1%	NS
fd-blaA-L ₁ ⁵		1×10^7	<0.1%	NS
fd-blaA-L ₁ ⁶		3×10^5	<0.1%	NS
fd-blaA-L ₁ ^R L ₂ ⁶	G ₄₁ A $\rightarrow$ (X) _{6–9} and G ₂₃₈ ER $\rightarrow$ (X) ₃	1.6×10^6	<0.1%	NS
fd-blaA-L ₁ ^R L ₂ ⁷		2.2×10^6	<0.1%	NS
fd-blaA-L ₁ ^R L ₂ ⁸		1.2×10^6	<0.1%	NS
fd-blaA-L ₁ ^R L ₂ ⁹		1.7×10^6	<0.1%	NS
fd-blaA-L ₁ ^R L ₂ ^{C5C}	G ₄₁ A $\rightarrow$ C-(X) _{5–7} -C and G ₂₃₈ ER $\rightarrow$ (X) ₃	2.9×10^6	4.9%	388 sec^{-1}
fd-blaA-L ₁ ^R L ₂ ^{C6C}		1.3×10^6	8.6%	287 sec^{-1}
fd-blaA-L ₁ ^R L ₂ ^{C7C}		5.1×10^6	4.7%	251 sec^{-1}
fd-blaA-L ₁ ^R L ₂ ^{C5C} L ₃ ⁶	G ₄₁ A $\rightarrow$ C-(X) _{5–7} -C	8×10^6	7%	239 sec^{-1}
fd-blaA-L ₁ ^R L ₂ ^{C6C} L ₃ ⁶	G ₂₃₈ ER $\rightarrow$ (X) ₃	2×10^8	3.5%	
fd-blaA-L ₁ ^R L ₂ ^{C7C} L ₃ ⁶	and T ₂₇₁ $\rightarrow$ (X) ₆	9×10^7	6.7%	

(NS) Not selected.

mutations (Huang et al. 1996). We tested its tolerance to insertions by replacing the dipeptide Gly41-Ala42 by six, seven, eight, or nine random residues (libraries fd-blaA-L₁^RL₂^{6–9}). Loop 3 (L3), between strand β 5 and the C-terminal helix α 11, has been previously engineered in our laboratory by replacing residue Thr271 by six random residues, and the insertion was shown to be rather well tolerated (Legendre et al. 1999). The three insertion points lie $\sim 12 \pm 1$ Å apart, defining a nearly equilateral triangle. Glu240 is closest to the active site Ser70 (distance between Ser O γ and Glu C=O, 12 Å), the others being 18 Å and 22 Å away, respectively.

Construction and properties of libraries

The following strategy was used for the construction of the libraries. The previously described phage fd-bla (Soumilion et al. 1994) was chosen as vector so as to allow, at a later stage, the selections of mutants that have acquired an affinity for target ligands (see accompanying paper in this issue by Mathonet et al.). Unique restriction sites were introduced within the β -lactamase gene together with a large deletion between these sites to avoid contamination of the libraries by the wild-type phage-enzyme, which would otherwise prevent the determination of insertion tolerance by simple selection for activity. The libraries were constructed by cloning PCR fragments obtained with degenerated primers.

In the first libraries created (fd-blaAL₁^{3–6}), residues 240 and 241 in loop L1 were replaced by three, four, five, or six random residues. The diversity of the libraries was defined as the number of transformed cells after electroporation and sequencing of randomly picked clones (Table 1). The acceptability of inserts was then assessed by determining the percentage of clones conferring

ampicillin resistance to bacteria grown on medium containing 20 $\mu\text{g/mL}$ of ampicillin, corresponding to $\sim 5\%$ of the maximal concentration on which cells infected by the wild-type phage-enzyme can grow. Less than 0.1% of the clones from libraries fd-blaAL₁^{3–6} generated transformed cells that grew on this selective medium. Some active clones were sequenced and most showed deletion of the inserted peptide, leaving only two residues, even when only a tripeptide insertion was attempted (Table 2). Libraries fd-blaAL₁^{3–6} were also selected on cefotaxime (0.03–4 $\mu\text{g/mL}$) and ceftazidime (0.12–16 $\mu\text{g/mL}$), since amino acid substitutions in loop L1 have been shown to generate mutants with increased hydrolytic activity against these extended spectrum antibiotics (Raquet et al. 1994; Venkatachalam et al. 1994; Cantu et al. 1996). Colonies developed only at the lowest antibiotic concentrations. A few phage-enzymes were characterized further and were found to have very low cephalosporin hydrolytic activity. We concluded that insertions in loop L1 are not tolerated by the enzyme.

An alternative strategy was therefore explored in which the residues in loop L1 were randomized in order to create diversity without insertion. In the second pool of libraries created (fd-blaA-L₁^RL₂^{6–9}), residues Gly238-Glu240-Arg241 were replaced by three random residues. In order to introduce diversity without excessive disruption of catalytic activity, the residues substituted at each position were determined on the basis of the results of the saturation mutagenesis experiments of Huang et al. (1996). Gly238 was replaced by Ala, Gly, Cys, or Ser, whereas Glu240 and Arg241 were replaced by all 20 possible residues. The engineering of loop L2 was performed at the same time as the random replacement in loop L1, with residues 41 and 42 being replaced by six, seven, eight, or nine random residues. The diversity of

Table 2. Sequences at position 240–241 of mutants isolated from the *fd-blaA-L₁^{3–6}* libraries resistant to different ampicillin concentrations

Library	Amp ₀	Amp ₂₀	Amp ₅₀	Amp ₁₀₀
fd-blaA-L ₁ ³	T-R-P	H-N (3)	H-N (5)	H-N (3)
	A-A-R	G-G		
	A-P-Q			
	A-L-E			
	X-Q-P			
fd-blaA-L ₁ ⁴	T-F-Q-Q	H-A	E-R (2)	E-R (5)
	S-H-Y-T	T-Q	G-N (2)	
	G-H-V-E	E-R	A-F	
		T-E		
fd-blaA-L ₁ ⁵	S-E-P-T-V	E-C	E-R (3)	
	L-N-E-A-N	E-R		
	N-P-R-T-K	H-D-S-N-G (2)		
	P-R-D-P-H	E-D-A-N-G		
fd-blaA-L ₁ ⁶	W-E-Q-Q-V-P		A-Q	X-H
	T-X-S-Y-X-H		V-L	
	C-P-E-Q-X-P		A-Q	
	Q-X-Q-H-R-P		X-A	
	T-G-N-E-G-Y		X-X	
	P-K-S-S-D-S			
	T-S-L-E-P-K			
	X-T-R-A-X-E			
	Q-R-V-G-W-H			
	K-P-S-L-A-V			
	R-L-N-Stop-R-R			

Amp_n indicates *n* mg/L ampicillin. The numbers in parentheses indicate the number of times the same sequence was found. The wild-type sequence is shown in bold. (X) Not readable.

these libraries is indicated in Table 1. Again, <0.1% of the clones conferred resistance to 20 μg/mL of ampicillin. Some active clones were sequenced and, interestingly, in libraries with insertions of seven, eight, or nine residues, each active mutant carried cysteines on both side of the inserted peptide (Table 3).

Given this observation, these libraries were rebuilt with cysteines encoded in the first and last position of the loops, and 4.7%–8.6% of the clones from libraries

fd-blaA-L₁^RL₂^{C5C→C7C} allowed transformed bacteria to grow on 20 μg/mL of ampicillin. Loop L2 therefore appears to accept insertions containing more than six residues, but only in the presence of these two cysteines.

The active libraries containing the random replacement in loop L1 and the insertion of five, six, or seven residues between two cysteines in loop L2 (*fd-blaA-L₁^RL₂^{C5C→C7C}*) were then combined with the active library containing the insertion of six random residues in loop L3 (Legendre et al. 1999). Together, these second-generation libraries (*fd-blaA-L₁^RL₂^{C5C→C7C}L₃⁶*) contained ~2.9 × 10⁸ clones, with a high percentage of active clones (Table 1). Clones picked at random before and after selection for activity were sequenced. From the 28 clones sequenced from the naïve collection (i.e., not selected for activity), only one was incorrectly built, containing a mutated residue instead of an insertion in loop L3. The remaining 27 carried the three engineered loops. From the collection selected for activity, 120 mutants were sequenced; all carried the random replacement in loop L1 and the insertion in loop L2, but only eight had a 6-residue insertion in loop L3. Three clones contained a smaller insertion, while the remaining 108 carried either a mutated residue at 271 (30 clones) or the wild-type threonine residue (78 clones) (Table 4; Fig. 2). Altogether, more than one million of the active properly folded mutants contained three engineered loops, which represents a relatively high diversity.

Distribution of residues in loops

Since numerous ampicillin-resistant clones were sequenced, an analysis could be performed of the amino acid preferences in the engineered loops, calculated as the observed occurrence divided by the theoretical diversity, i.e., that expected from the relative numbers of codons specifying the corresponding residue. The normalized occurrences at defined positions are presented in Figures 3 and 4, in which the residues are arranged in order of decreasing propensity

Table 3. Sequences at positions 41–42 and 240–241 of mutants isolated from the *fd-blaA-L₁^RL₂^{6–9}* libraries resistant to 100 mg/L of ampicillin

Library	Mutant	Sequence at 41–42 (L ₂)	Sequence at 238–241 (L ₁)
fd-blaA		G-A	G-E-R
fd-blaA-L ₁ ^R L ₂ ⁶	#1	S-A-K-A-R-A	G-D-H
	#2	A-A-T-G-H-A	G-E-N
fd-blaA-L ₁ ^R L ₂ ⁷	#1	C-T-P-G-Q-S-C	G-L-N
	#2	C-S-T-A-D-N-C	A-P-H
fd-blaA-L ₁ ^R L ₂ ⁸	#1	C-A-L-S-G-R-Y-C	G-L-N
	#2	C-V-R-E-V-Q-S-C	G-Q-N
	#3	C-R-R-R-D-V-N-C	G-A-Q
fd-blaA-L ₁ ^R L ₂ ⁹	#1	C-M-D-A-A-V-P-Y-C	G-E-A
	#2	C-R-R-V-A-G-K-V-C	G-L-Q

Table 4. Sequences of clones isolated from the $fd\text{-}blaA\text{-}L_1^R L_2^{CXC} L_3^6$ libraries resistant to 20 mg/L of ampicillin

Library	Clone	Sequence at 41–42 (L_2)	Sequence at 238–241 (L_1)	Sequence at 271 (L_3)	Additional mutations
$fd\text{-}blaA$		G-A	G-E-R	T	
$fd\text{-}blaA\text{-}L_1^R L_2^{C5C} L_3^6$	Amp ₂₀ -#3	C-Y-N-A-G-S-C	G-G-N	R-V-T	
	Amp ₂₀ -#20	C-R-T-L-I-H-C	G-L-N	T-G	$A_{270} \rightarrow E$
$fd\text{-}blaA\text{-}L_1^R L_2^{C6C} L_3^6$	Amp ₂₀ -#2	ND	G-K-M	T-G-R-H-A-L	
	Amp ₂₀ -#7	C-V-X-G-G-Q-M-C	G-A-Y	A-P-Y-A-G-G	
	Amp ₂₀ -#12	C-A-G-L-A-T-A-C	G-R-N	G-E-R-G-A-S	
	Amp ₂₀ -#18	C-R-A-H-F-A-N-C	G-T-R	G-R-A-G-A-G	
	Amp ₂₀ -#28	C-G-V-H-W-R-S-C	G-L-N	A-S	
	Amp ₂₀ -#31	C-X-S-Q-H-D-V-C	G-R-Q	G-G-T-V-T-G	
	Amp ₂₀ -#32	C-I-A-M-S-S-S-C	G-G-R	T-R-Q-G-S-N	
$fd\text{-}blaA\text{-}L_1^R L_2^{C7C} L_3^6$	Amp ₂₀ -#8	ND	X-X-R	E-N-S-E-X-T	$M_{272} \rightarrow Q$
	Amp ₂₀ -#20	C-V-R-S-A-I-H-S-C	G-R-N	G-K-D-F-K-N	

(ND) Not determined.

of presence in Ω -loops of high accessibility computed from a large database (Pal and Dasgupta 2003). The residues are also grouped as high (1.6), medium (0.9), or low (0.5) mean Ω -loop preference, represented by the white, gray, and black bars.

Loop L1 (residues 238–241)

The accepted residues at positions 238–241 in loop L1 are shown in Figure 3. Only glycine, alanine, cysteine, and serine were tested in position 238 as suggested by the results of Huang et al. (1996). Glycine, the residue present in the wild-type enzyme and more generally in class A β -lactamases, was overwhelmingly represented in this position; alanine and serine residues were observed, but were clearly disfavored, and no cysteine was found in any of the 124 sequenced clones. Mutations are often observed in this position in β -lactamase mutants active against third-generation cephalosporins (Lee et al. 1991; Palzkill and Botstein 1992; Cantu et al. 1996).

Since codons 240 and 241 were replaced by NN(G/T), all residues were permitted in these positions. Nearly all amino acids were observed, but hydrophobic residues were rare. Proline was accepted at residue 240, but almost completely rejected at residue 241. While the ϕ/ψ angles of Glu240 are compatible with the constraints of proline, the values observed for Arg241 are not allowed for this residue and a change in the conformation of the turn would be required to accommodate it. Lysine was also selected at position 240 for replacement of the wild-type glutamic acid. This mutation is often present in TEM β -lactamase mutants that have evolved to be active on extended spectrum antibiotics (Raquet et al. 1994). Mutant E240K was characterized by Cantu et al. (1996) and its activity on ampicillin was found to be half of that of the wild-type enzyme. However, this mutation has been shown to stabilize the enzyme (Raquet et al. 1995), which may explain why it was frequently selected at position 240 from our libraries. Asparagine and basic residues

were selected at position 241. Overall, these results agree with those of Huang et al. (1996), if we take into account the fact that their selection pressure for wild-type-like activity was higher than ours.

Loop L2 (residues 41–42)

The insertion in loop L2 was performed by replacing residues 41–42 by five, six, or seven residues, flanked by two cysteines. The 20 amino acids were encoded by NN(G/T) codons. The numbering of the positions was defined as follows:

---Leu₄₀-Cys-Xaa-Xaa-(Xaa)_{1,2, or 3}-Xaa-Xaa-Cys-Arg₄---
position : 1 2 Mid $n-1$ n

Figure 4A shows the amino acid distribution at each position. Again, nearly all residues were found. Overall, glycine, alanine, serine, and valine residues were favored. Lysines and tryptophans were relatively disadvantaged

Figure 2. Characteristics of combination library $fd\text{-}blaA\text{-}L_1^R L_2^{CXC} L_3^6$. Twenty-eight clones from the total library of 2.9×10^8 clones and 120 from the active library of 1.35×10^7 clones were sequenced. The color code is as follows: (1) two complete inserts in L2 and L3 (2.8×10^8 clones in the total library, 10^6 clones in the active library); (2) a complete insert in L2 and an insert smaller than six residues in L3; (3) a complete insert in L2 and a mutated residue in L3; (4) a complete insert in L2 and a nonmutated T271 in L3. Residues in L1 are mutated. The inner circle is the fraction of the library corresponding to the active clones. It is magnified for easier observation of the various types of clones in this portion of the combination library.

Figure 3. Relative amino acid occurrences in the three positions of loop L1 (according to the Ambler numbering; residue 239 is missing in TEM-1 β -lactamase). The relative occurrence was calculated as the ratio of the observed occurrence (x_{obs}) to that expected from the total number of observations (n = number of clones sequenced), given the number of codons coding for a particular residue (x_{exp}).

and unique cysteines were never observed as usual for phage libraries (Kay et al. 1993).

Loop L3 (residue 271)

The insertion in loop L3 was performed by replacing Thr271 by six residues (Legendre et al. 1999). All 20

amino acids were tested. The numbering of the positions was defined as follows:

As shown in Figure 4B, most residues were tolerated in the L3(2) and L3(Mid) positions, although hydrophobic residues were less frequently observed than in loop L2. At the ends of loop L3, marked preferences were noticed: The wild-type threonine or its analog serine appeared clearly favored.

Effect of the disulfide bridge in loop L2 on activity and stability

Since the presence of two cysteines appeared to be required to generate active clones after introduction of insertions in loop L2, and since this suggested that a disulfide bridge is involved in the stabilization of these enzymes, we decided to express two clones derived from the fd-blaA-L₁^RL₂^{CxC} libraries as free enzymes and to characterize them better. The sequences in loops L1 and L2 were the following: for clone J1, L1 = AAN and L2 = CGADSKTQC; for clone J2, L1 = GDT and L2 = CRSTLGAGC. Their specific activity was measured with PenG as substrate: k_{cat} values of 89 sec⁻¹ and 416 sec⁻¹ were obtained, to be compared with a k_{cat} of 1500 sec⁻¹ for the wild-type enzyme.

Determination of free thiol groups with Ellman's reagent with or without denaturant indicated no free -SH group, while glutathione was easily titrated at the same concentration. The stability of these mutants' reversible denaturation was determined by measurement of the fluorescence intensity as a function of guanidinium hydrochloride concentration, [Gu.HCl]. In the absence of reducing agent, sigmoidal transitions were observed with midpoints around 1.1 M for mutant J1 and 0.9 M for mutant J2. Equilibrium constants of denaturation (K_D) were extracted from these curves. Plots of a ΔG_D° versus [Gu.HCl] were linear. Extrapolation to zero Gu.HCl concentration gave $\Delta G_{D,H_2O}^\circ$ values of 18.3 ± 1.2 and 14.8 ± 1.2 kJ/mole with slopes of $-(17.2 \pm 1.1)$ and $-(16.6 \pm 1.4)$, respectively, for mutant J1 and J2 (means of two experiments). These values are to be compared with those reported for the same transition in the wild-type enzyme: $\Delta G_{D,H_2O}^\circ$ of 21.7 ± 1.7 kJ/mol and $-(24.2 \pm 1.7)$ (Vanhove et al. 1997). The mutants appear to be destabilized by 3–7 kJ/mole. After 6 h incubation in

Figure 4. Relative amino acid occurrences in the extended loops L2 (A) and L3 (B). The relative occurrence was calculated as the ratio of the observed occurrence (x_{obs}) to that expected from the total number of observations (n = number of clones sequenced), given the number of codons coding for a particular residue (x_{exp}). The amino acids are arranged in order of decreasing propensity of presence in Ω -loops of high accessibility computed from a large database (Pal and Dasgupta 2003) and are shown as three groups of high, medium, and low mean Ω -loop preference, indicated by white, gray, and black bars. The significance of the deviation of the $x_{\text{obs}}/x_{\text{exp}}$ ratio is shown as significant (*), highly significant (**), or very highly significant (***), with the probability of a chance observation being 5%, 1%, and 0.1%, respectively (see Materials and Methods).

Figure 4. (Legend on previous page)

25 mM DTT, the fluorescence intensity of mutant J1 at 0 M in Gu.HCl strongly decreased compared with untreated protein. Measurement of the fluorescence intensity as a function of Gu.HCl concentration did not allow detecting a sigmoidal signal, but only a monotonous decrease. The effect of DTT on the β -lactamase activity of the purified proteins was also assessed. The relative activity of the mutants (ratio of the catalytic activities with and without reducing agent) decreases slowly with time ($t_{1/2} \approx 3\text{--}4$ h).

Discussion

Despite all of the progress made in structural studies on proteins, the effect of insertions in loops or turns on structural stability remains difficult to predict (Collinet et al. 2001; Batori et al. 2002). An increase in loop length can induce a decrease in the global stability of the protein as a consequence of the increase in entropic cost in bringing together the secondary structures connected by the loop. This effect has been clearly demonstrated in studies in which mainly polyglycine peptides were inserted in loops of variable rigidity (Ladurner and Fersht 1997; Nagi and Regan 1997; Viguera and Serrano 1997).

In the work reported here, we attempted to insert random peptide sequences within rather rigid loops. As in the examples mentioned above, a destabilizing entropic contribution was expected, although this might be lower than with polyglycine peptides, as the conformational freedom of other residues is lower than that of glycine in the denatured state. On the other hand, enthalpic contributions arising from interactions between residues within the loop or with the rest of the protein may stabilize or further destabilize the structure (Tramontano et al. 1989). The complexity of these effects makes prediction particularly hazardous. The examination of the behavior of libraries is, however, likely to give a view on the effects of insertions that is different from that obtained in biophysical studies of individual mutants, although, in this case, no thermodynamic analyses will be available, as there is no good criterion to choose the mutants to be investigated.

In order to assess insertion tolerance and find sequences compatible with folding, it is essential to have a robust screen for functionality. We relied on a selection protocol based on enzymatic activity. On the positive side, this protocol ensures that the selected enzymes are indeed properly folded and sufficiently stable to survive any *in vivo* proteolysis. However, some folded proteins can be inactive because of interference by the inserted residues with substrate binding or catalysis. This is likely to be the case for the insertions in loop L1 bordering the active site. Insertions in the other two loops are less likely to affect the activity directly, even if long-distance effects on activity cannot be excluded. Indeed, a perturbation resulting from

binding of an “allosteric effector” between helices 10 and 11, 16Å from the center of the active site, has been shown to inhibit the enzyme (Horn and Shoichet 2004).

This work showed that there was little relationship between tolerance to mutations and to insertions. Both sequence alignment and random mutagenesis experiments indicate that loop L1 is rather tolerant to mutations, especially in positions 240 and 241 (Huang et al. 1996). Furthermore, an insertion or deletion of one residue has been naturally accepted in the class A β -lactamases. We observed that, in general, insertions in this position were not compatible with activity. Insertions in loops L2 and L3 transformed relatively rigid three- or six-residue loops, connecting strands, and helices into medium size Ω -loops. Loops L2 and L3 were only moderately tolerant of mutations, but their tolerance to insertion was quite high provided stabilization was afforded by additional constraints. The requirement for the presence of two cysteines at the ends of loop L2 suggested the creation of a disulfide bridge, which was confirmed by biochemical analyses on two mutants expressed as free proteins. The use of a disulfide bridge as source of stabilization of a library of inserted peptides has been implemented earlier by insertion in the disulfide-constrained bridge of the thioredoxin active site (Lu et al. 1995). For obvious reasons, the compatibility of insertions with activity was not determined. A weaker constraint was noticed in loop L3: On replacement of Thr271 by a random hexapeptide, it was observed that a threonine or a serine residue was very frequently present at the N or C terminus of the insert. This presumably allowed conserving two rigidifying hydrogen bonds present in the wild-type enzyme between the hydroxyl function of Thr271 and the O ϵ 1 and NH of Glu274. Both constraints suggest possible solutions for increasing the acceptability of inserts within an enzyme scaffold without compromising the activity too much. A relationship between mobility and insertion tolerance has been suggested in a study on the effects of insertions in helices of the T4 lysozyme (Vetter et al. 1996). The fact that insertion is tolerated in two relatively rigid loops might suggest an exception to this rule, but the two mechanisms described above could explain the apparent exception; both might contribute to restore some of the rigidity that was present in the original loop.

An observation of the effect on activity of di- or pentapeptide insertions in random positions of the TEM β -lactamase and Cre recombinase (Barany 1985; Hallet et al. 1997; Petyuk et al. 2004) confirms the absence of general correlation between mutation and insertion tolerance, as suggested by our results. Indeed, with both enzymes, insertions appear to be tolerated in several positions where residues are highly conserved, naturally or in saturation mutagenesis experiments. Conversely, inactivating insertions have been observed between

mutation-tolerant residues (e.g., Glu63 in another β -lactamase loop). In each case, tentative explanations can be presented on the basis of detailed analyses of the structures.

One surprising observation was the requirement for the presence of two cysteines in loop L2, but not in loop L3. Both loops are relatively short and rigid and, at first sight, symmetrically positioned, respectively, between the N-terminal helix α_1 and the first strand β_1 and between the last strand β_5 and the C-terminal helix α_{11} . Loop L2 is made up of four residues (41–44), while loop L3 contains six residues (266–271) and its mobility is slightly greater, as estimated from crystallographic B-factors (Jelsch et al. 1993), but these slight differences may not be sufficient to explain the different behavior. Less obvious factors may be involved, namely, differences in the stability of the helices and their interactions with the rest of the protein. Secondary structure predictions for the N-terminal helix of TEM-1 β -lactamase give different results depending on the method used, whereas the structure of the C-terminal helix is always predicted (e.g., Combet et al. 2000). Moreover, if the force-field energy (FFE) is calculated for both helices extracted from the structure, the N-terminal helix is unstable (FFE = +62.2 kJ/mol), while the C-terminal helix is stable (FFE = –667.0 kJ/mol) (Guex and Peitsch 1997). The energy of interaction with the rest of the protein (estimated from the difference between the total FFE and the sum of the FFEs of the fragments) is 36% greater for the C-terminal helix, which may reflect the difference in length between the two helices (13 vs. 17 residues). A similar explanation of differences in secondary structure stability has been proposed by Gu et al. (1997) to explain the contrasting effects of random mutageneses on two symmetrically disposed β -turns on the folding and stability of the peptostreptococcal protein L: The turn with the lowest percentage of functional variants is located between two strands, one of which has a low strand-forming propensity score. In the case of the β -lactamase, the difference in behavior may reflect a fundamental property of the protein, since β -lactamases must be exported, and this translocation requires an ordered unfolding process in which the amino-terminal is expected to be unfolded first (Prakash and Matouschek 2004).

A combinatorial library in which the three loops were engineered was created in a hierarchical way. First-generation libraries created independently and selected for β -lactamase activity were combined to create a large second-generation library. This hierarchical construction allows a suitable control of library quality, since only properly folded mutants have enzymatic activity. In the unselected combination library, 96% of the mutants contained mutations in loop L1 and programmed inserts in loops L2 and L3, whereas, after selection for activity, only 7% of the active mutants had insertions in both loops

L2 and L3. Zhou et al. (1996) have shown that randomization of a loop sequence generates a high proportion of properly folded mutants only if the host protein is stable. It appears that this rule can be extended to insertions. The introduction of the first insertion is likely to be destabilizing for most mutants. This prediction was confirmed with the two mutants investigated in more detail, which are less stable versus Gu.HCl-induced denaturation than the wild-type enzyme. Since most proteins coded by the clones in the individual libraries might already be destabilized by the first insertion, a second insertion would only be accepted if it had a low destabilizing effect and a decreased diversity of sequences in the loops would be expected. This effect was seen, but mainly in loop L3, probably because of the stabilization of loop L2 by the disulfide bridge. The implementation of additional sequence constraints may offer a way of decreasing the destabilization. Indeed, in the eight mutants sequenced from the combinatorial library in which both loops L1 and L3 had the programmed insertions, 28% of the residues were glycine. This suggests that, when the perturbation of the enzyme is increased by introduction of insertions in two positions, it becomes necessary to introduce more flexibility in some positions. Ladurner and Fersht (1997) also observed that the insertion of alanine- or glutamine-containing peptides in the mobile loop of CI2 were more destabilizing than the insertion of the same number of glycine residues.

Sequence analyses indicate that the occurrence of hydrophobic residues was significantly higher in our engineered Ω -loops than in Ω -loops of natural proteins (Pal and Dasgupta 2003). The amino acid preference or avoidance was somewhat different in loops L2 and L3, with hydrophobic residues being less frequent and threonine or serine strongly favored in position 1 or n of loop L3. The observed occurrence of a large variety of residues is of major interest for the creation of binding sites based on loops (see accompanying paper in this issue by Mathonet et al.).

Materials and methods

General

Penicillin G, ampicillin, tetracycline, DTNB, and DTT were purchased from Sigma. Restriction endonucleases, Taq polymerase, and T4 DNA ligase were obtained from Roche Molecular Diagnostics.

Oligonucleotides

The synthetic oligonucleotides were from Eurogentec (restriction sites are underlined, mutations are in bold):

O1: 5'-GGA-GGG-CTT-ACC-ATC-GGG-CCC-CAG-TGC-TGC-A-3';

O2: 5'-TGC-AGC-ACT-GGG-GCC-CGA-TGG-TAA-GCC-CTC-C-3';

O3: 5'-TCG-AGT-GGG-TTA-CAT-CGA-ACT-GGA-AGA-CGG-GTC-TCG-CGG-TAT-CAT-TGC-AGC-ACT-GGG-GCC-3';
 O4: 5'-CCA-GTG-CTG-CAA-TGA-TAC-CGC-GAG-ACC-CGT-CTT-CCA-GTT-CGA-TGT-AAC-CCA-C-3';
 O5: 5'-TGC-ACA-GCC-AGA-AAC-GCT-GGT-GAA-AGT-AAA-AGA-TGC-TGA-AGA-TCA-GGT-CTT-CGC-3';
 O6: 5'-TCG-AGC-GAA-GAC-CTG-ATC-TTC-AGC-ATC-TTT-TAC-TTT-CAC-CAG-CGT-TTC-TGG-CTG-3';
 O7: 5'-CAG-TTG-GGT-GCT-CGA-GTG-GGT-TAC-ATC-G-3';
 O8-O11: 5'-GTC-GAA-ACT-CGA-AGA-CGC-AGA-CCC-(MNN)_n-ACC-GGC-TCC-AGA-TTT-ATC-AGC-AAT-AAA-CC-3' where *n* is 3, 4, 5, or 6;
 O12-O15: 5'-CAG-CTT-TGA-GGA-AGA-CAA-GAT-CAG-TTG-(NNK)_n-CGA-GTG-GGT-TAC-ATC-GAA-CTG-GAT-CTC-AAC-AGC-G-3', where *n* is 6, 7, 8, or 9;
 O16: 5'-GTC-GAA-ACT-CGA-AGA-CGC-AGA-CCC-MNN-MNN-RSM-GGC-TCC-AGA-TTT-ATC-AGC-AAT-AAA-CCA-GCC-3';
 O17-O19: 5'-CAG-CTT-TGA-GGA-AGA-CAA-GAT-CAG-TTG-TGC-(NNK)_n-TGC-CGA-GTG-GGT-TAC-ATC-GAA-CTG-GAT-CTC-AAC-AGC-G-3'; where *n* is 5, 6, or 7.
 O20: 5'-TTC-TGT-ATG-AGG-TTT-TGC-TAA-3';
 O21: 5'-GCG-GGG-TAC-CTC-ATG-AAA-AAA-TTA-TTA-TTC-G-3';
 O22: 5'-T-GCG-CTG-CAG-TTC-TAG-ACC-CCA-ATG-CCT-AAT-C-3'.

Construction of the libraries

Construction of cloning vectors

Three cloning vectors were derived from the filamentous fd phage carrying the TEM-1 β -lactamase gene fused with the coat protein pIII gene (fd-bla; Soumilion et al. 1994). Fd-blaA features a unique ApaI restriction site at position encoding residues 251–252 in the β -lactamase gene (between positions encoding libraries L1 and L3). It was created by site-directed mutagenesis with oligonucleotides O1 and O2 using the “Quick Change Site-Directed Mutagenesis Kit” from Stratagene. The resulting mutation is silent.

In fd-blaA- Δ 1, nucleotides encoding residues 50–241 of the functional *bla* gene were replaced by six base pairs constituting a BbsI restriction site. The complementary oligonucleotides (O3 and O4) containing the BbsI site were annealed and cloned between the XhoI and ApaI restriction sites of fd-blaA.

Fd-blaA- Δ 2 was created to introduce another BbsI restriction site in fd-blaA- Δ 1 at a position encoding residues 40–41. The oligonucleotides (O5 and O6) containing the second BbsI site were annealed and cloned between the ApaI and XhoI restriction sites of fd-blaA- Δ 1.

Construction of libraries

fd-blaA-L₁^{3→6}. Four cloning cassettes were constructed by PCR with, on one side, the primer O7 and, on the other, four degenerated primers (O8–O11) that allow the replacement of residues 240 and 241 of TEM-1 β -lactamase by three, four, five, or six random residues. These primers also contain a BbsI site. Vector fd-blaA was used as template to recover the deleted part of the *bla* gene. After purification on low-melting agarose or using the High Pure PCR Product Purification Kit (Roche), the cassettes were digested by XhoI and BbsI and repurified. They were used at a fivefold excess in a ligation reaction with the fd-blaA- Δ 1 vector, which had been previously XhoI and BbsI restricted and purified on ‘Chroma Spin 1000’ (Clontech). The contaminating cloning vector was removed by BbsI digestion and the ligation mixture used for the transformation of *Escherichia coli* TG1 cells by electroporation.

fd-blaA-L₁^RL₂^{6→9}. The libraries in which residues 41–42 were replaced by six to nine random amino acids and residues 238–240–241 were randomized, were constructed by PCR using the degenerate primers O12–O15 and O16, which also contain the BbsI site. The deleted part of the *bla* gene was recovered from fd-blaA. After purification on low-melting agarose, the cassettes were digested by BbsI and repurified using ‘Chroma Spin 200’ (Clontech). These cassettes were used as a fivefold excess in a ligation reaction with the fd-blaA- Δ 2 vector, previously BbsI restricted and purified on ‘Chroma Spin 1000’ (Clontech). The contaminating cloning vector was removed by BbsI digestion and the ligation mixture used for the transformation of *E. coli* TG1.

fd-blaA-L₁^RL₂^{C5C→C7C}. The libraries with the two cysteines bordering the inserts were constructed as above. Oligonucleotides O17, O18, and O19 were used to introduce diversity into region 41–42. The BbsI restriction site is underlined and the cysteine codons are shown in bold and underlined.

fd-blaA-L₁^RL₂^{C5C→C7C}L₃⁶. Library lib3B encoding the replacement of Thr271 by six random residues has been described previously (Legendre et al. 1999). The library selected for catalytic activity was amplified by PCR using oligonucleotides O20 and O2 (which introduces an ApaI restriction site). After purification by phenol extraction, the PCR fragment was digested by NotI and ApaI and repurified (phenol extraction). This cassette was used at a three- or fivefold excess in a ligation reaction with the fd-blaA-L₁^RL₂^{C5C→C7C} vectors, previously NotI and ApaI restricted and purified on ‘Chroma Spin 1000’ (Clontech). The ligation mixture was finally purified (phenol extraction) and transformed into *E. coli* TG1.

Phage-enzyme production and purification

Phage-enzyme libraries were produced by spreading electro-transformed bacteria on 530-cm² plates of solid LB-tet medium (LB containing 7.5 μ g/mL tetracycline; fd-bla includes a tetracycline resistance gene in the intergenic region). Transformants were grown overnight at 37°C and recovered by washing the plates with liquid LB medium. For the second-generation libraries (fd-blaA-L₁^RL₂^{C5C→C7C}L₃⁶), the transformed bacteria were transferred to liquid LB-tet medium and grown overnight at 37°C. In both cases, bacteria were removed by centrifugation and the phage purified from the supernatants by polyethylene glycol/NaCl precipitations. Libraries selected for activity were produced under the same conditions, except that they were plated on medium containing 20 μ g/mL ampicillin.

Phages from libraries or from individual clones were amplified at 23°C for 60 h in *E. coli* TG1 in liquid LB-tet medium, recovered from the culture supernatant by two successive polyethylene glycol precipitations, and resuspended in TBS (150 mM NaCl, 50 mM Tris at pH 7.5). Between the first and second precipitations, the phage suspension was filtered through a 0.45- μ m filter. Stock phage concentrations were determined by measuring the absorbance at 265 nm using 8.4×10^7 M⁻¹ cm⁻¹ as the extinction coefficient.

Characterization of libraries

Kinetic measurements

The β -lactamase activity of phage-enzymes was determined at room temperature in 50 mM phosphate buffer (pH 7.5). The specific activity of the phage-displayed enzymes was calculated from complete progress curves of product

formation using penicillin G and measuring the change in absorbance as a function of time at 232 nm ($\Delta\epsilon = 1042 \text{ M}^{-1} \text{ cm}^{-1}$). The first-order rate constant, k_{cat} (turnover number), was calculated by dividing the V_{max} by the total phage-enzyme concentration, determined by measuring the optical density at 265 nm.

Sequence analysis and statistics

The sequences of the genes encoding the mutants were determined using the "DYEnamic ET Terminator cycle Sequencing Kit" from Amersham Pharmacia and the "ABI Prism BigDye Terminator Sequencing Ready Reaction Kit" from Applied Biosystems on a ABI PRISM 377 DNA sequencer from Applied Biosystems.

To determine whether the number of observations of an amino acid (x_{obs}) in a given position deviated significantly from the expected mean (x_{exp}), we used the binomial law to calculate the probability of this occurring by chance as :

$$P_{x_{\text{obs}}} = C_N^{x_{\text{obs}}} \cdot p^{x_{\text{obs}}} \cdot (1-p)^{N-x_{\text{obs}}}$$

where

$$C_N^{x_{\text{obs}}} = \frac{N!}{x_{\text{obs}}! \cdot (N - x_{\text{obs}})!},$$

where N is the total number of observations and p the ratio between the number of codons encoding the specific amino acid (c) and the total number of codons (C).

Expression and characterization of proteins

Two mutants, J1 (L1 sequence, AAN; L2 sequence, CGADSKT QC) and J2 (L1 sequence, GDT; L2 sequence, CRSTLGAGC), were expressed as free proteins (with Myc and His tags). Genes were recovered by PCR using oligonucleotides O21 and O22 and cloned between the NcoI and XbaI sites of an expression vector derived from pBAD/Myc-His (Invitrogen), in which the Amp^R gene had been replaced by a Tet^R gene (a gift from Dr. Bernard Hallet, Unité de génétique, Université catholique de Louvain). The proteins were purified by elution from a Ni-chelating column (HiTrap chelating HP from Amersham Biosciences) in 20 mM HEPES buffer containing 0.5 M imidazole and dialyzed.

The amount of free sulfhydryl groups was measured by reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). The protein sample (1 μM) was mixed with DTNB (1 mM) in 50 mM phosphate buffer (pH 8.0), in the presence or absence of Gu.HCl (1 M). Glutathione solutions in the same buffer were used as reference. The absorbance was recorded at 412 nm.

The stability versus denaturation was measured by incubation of the purified proteins for 6 h at room temperature in 50 mM of filtered sodium phosphate buffer (pH 7.0), with or without 25 mM DTT. Unfolding of the protein (0.1 μM) induced by Gu.HCl (Sigma) was then monitored by following the changes in tryptophan fluorescence. The intensity of fluorescence emission was measured on a spectrofluorometer at 360 nm for mutant J1 and 350 nm for mutant J2 using an excitation wavelength of 280 nm. Denaturation curves were constructed by plotting the fluorescence versus the molar concentration of denaturant, [Gu.HCl]. The state of equilibrium was expressed in terms of an equilibrium constant, $K_D = (F - F_N)/(F_D - F)$, where F is the

measured fluorescence and F_N and F_D are the fluorescence of the protein in the native and denatured states, respectively. The free energy change on denaturation was calculated by using the relationship: $\Delta G_D^\circ = -RT \ln K_D$. ΔG_D° values were plotted against [Gu.HCl]. The plots were linear for each protein, and $\Delta G_{D,H_2O}^\circ$ and m were determined by least squares fittings: $\Delta G_D^\circ = \Delta G_{D,H_2O}^\circ - m [\text{Gu.HCl}]$, where $\Delta G_{D,H_2O}^\circ$ is the value of ΔG_D° at [Gu.HCl] = 0 M, and m is the slope of the straight line.

To measure the effect of DTT on activity, the purified proteins were incubated with 25 mM DTT at room temperature in 50 mM phosphate buffer (pH 7.0), the evolution of β -lactamase activity on PenG was followed during 24 h as described above.

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