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Effects of F171 Mutations in the 6′-*N*-Acetyltransferase Type Ib [AAC(6′)-Ib] Enzyme on Susceptibility to Aminoglycosides

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Substitutions at position F171 of 6′-*N*-acetyltransferase type Ib cause variable loss of aminoglycoside resistance, indicating that this residue plays an important role in the structure and/or function of the enzyme.

A common mechanism of resistance to aminoglycosides is enzymatic modification by acetyltransferases (11). Although detailed studies on these enzymes have been limited, some mechanistic and mutational studies have been carried out (reviewed in references 4 and 11) and the three-dimensional structures of aminoglycoside 6′-*N*-acetyltransferase type Ii and aminoglycoside 3-*N*-acetyltransferase type Ia have been reported (15, 18). The *aac(6′)-Ib* gene, included in the transposon Tn*1331*, encodes the 6′-*N*-acetyltransferase type Ib [AAC(6′)-Ib] enzyme, which confers resistance to several aminoglycosides (13, 14). We recently isolated a mutant with the F171L modification, which resulted in a protein with reduced activity at 42°C toward aminoglycoside molecules that contain a substitution at the C-1 amino group of the deoxystreptamine moiety (10). This change in specificity at a higher temperature suggests that the phenylalanine at position 171 is important for the structure and/or function of the enzyme (10). It has been shown by mutagenesis analysis that certain positions in proteins are quite tolerant of amino acid substitutions (2). However, those positions with low tolerance for substitutions were shown to be important for the function of the proteins either by playing a direct role in the activity or by being required for the proper three-dimensional structure or stability (12). For example, residues with low tolerance for substitutions were in general the most important for *lac* repressor activity (2). Therefore, we studied several mutations at F171 in AAC(6′)-Ib to determine this residue's tolerance for substitutions. Our results showed that amino acid F171 has a very low tolerance for amino acid substitutions, supporting the idea that F171 plays an important role in the activity or proper folding of AAC(6′)-Ib.

Methods. *Escherichia coli* XL1-Blue (Stratagene) was used as plasmid host. The plasmid pJHCMW1 (16) was used for the mutagenesis experiments. The mutant F171L was generated by in vivo mutagenesis (10), and the other mutants were generated with the QuikChange site-directed mutagenesis kit (Stratagene) following the recommendations of the supplier. Nucleotide sequencing was performed at the California State University Northridge Sequencing Facility. MICs were determined by the E-test method (17) with commercial strips (AB Biodisk).

Results. To determine the tolerance of AAC(6′)-Ib to substitutions at F171, we generated mutations to randomize the codon at position 171. Following mutagenesis, a random collection of cells harboring the mutant derivatives was plated onto L agar containing 30 μg of amikacin (AMK)/ml. DNA sequence analysis of several of the resultant colonies demonstrated that in all cases there was a phenylalanine residue at position 171, suggesting that the enzyme has a very low tolerance for substitutions at F171 if it is to remain capable of conferring resistance to AMK at this concentration on L agar plates. Following this, we selected colonies cultured in the absence of aminoglycosides and identified the amino acid encoded at position 171. *E. coli* strains carrying the plasmids harboring the mutated genes were analyzed by determining the MICs of several aminoglycoside antibiotics (Table 1). All nine mutant derivatives conferred substantially lower levels of resistance than did the wild type.

Comparison of two mutants with F171I (pCP220) and F171L (pDP1) substitutions indicated that, despite the very similar structures of leucine and isoleucine, they behaved differently not only from the wild type but also from each other. *E. coli* harboring the plasmid pCP220 (F171I mutant derivative) showed considerably lower MICs of AMK, kanamycin (KAN), netilmicin (NET), and tobramicin (TOB) than *E. coli*(pDP1) (Table 1). The ratios of the MICs of AMK, KAN, NET, and TOB for the mutant F171L to those for the mutant F171I were 8, 5.3, 6, and 1.5, respectively. Substitutions with the nonpolar amino acids tryptophan (pCP301) and methionine (pCP213) resulted in mutant derivatives that were able to confer detectable levels of resistance to AMK, KAN, NET, and TOB, although the levels were considerably lower than those conferred by the wild type (Table 1). *E. coli* harboring pCP224, the plasmid carrying the gene with a tyrosine substitution, showed a very low, albeit detectable, level of resistance to AMK, KAN, NET, and TOB (Table 1). The mutant derivatives F171G (plasmid pCP210), F171K (plasmid pSSF8), F171N (plasmid pSSF2), and F171S (plasmid pCP262) were unable to confer resistance against the tested aminoglycosides (Table 1).

Discussion. A thorough understanding of the structure and function of the AAC(6′)-Ib protein may play an important role in future rational drug design. To address this possibility we recently initiated an analysis of the *aac(6′)-Ib* gene by mutagenesis (10). This approach has been used to perform structure-function analysis of numerous proteins (1, 3, 5, 6, 9). In this paper, we show that amino acid substitutions at F171 result in enzyme derivatives that conferred substantially reduced or no resistance to various aminoglycosides. This low tolerance to substitutions suggests that F171 may be important for the enzymatic function either by playing a direct role in the activity or by being required for the proper three-dimensional structure or stability. F171 is one of the most conserved residues within a region designated as motif B in two subgroups of AAC(6′)

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TABLE 1. Susceptibility to aminoglycosides of plasmidless *E. coli* XL1-Blue and of the same strain harboring plasmids including the wild-type *aac(6′)-Ib* gene or mutant derivatives of the gene with substitutions for F171

Plasmid[a]	Amino acid 171	MIC (μg/ml)[b]				
		KAN	AMK	NET	TOB	GEN
None	N.A.[c]	0.5	0.25	0.19	0.25	0.064
pJHCMW1 (wild type)	Phe	128	24	64	32	0.75
pDP1	Leu	64	8	12	12	0.19
pCP213	Met	96	8	32	16	0.25
pCP301	Trp	32	4	4	16	0.5
pCP220	Ile	12	1	2	8	0.25
pCP224	Tyr	8	1.5	1.5	6	0.19
pCP210	Gly	3	0.75	0.75	2	0.19
pCP262	Ser	0.75	0.38	0.5	0.5	0.064
pSSF2	Asn	0.75	0.5	0.25	0.25	0.064
pSSF8	Lys	0.75	0.5	0.09	0.5	0.064

[a] Plasmid carried by *E. coli* XL1-Blue.
[b] MICs were determined by the E-test. GEN, gentamicin.
[c] N.A., not applicable.

enzymes (Fig. 1) (8, 11). Although the role played by this motif is still not clear (18), Lin et al. (7) have recently postulated that F174 [equivalent to AAC(6′)-Ib F171] in motif B of tGCN5 is part of the hydrophobic core and may be involved in binding acetyl coenzyme A. Elucidation of the three-dimensional structure of AAC(6′)-Ib will allow the determination of the role of F171 in the function and/or specificity of this enzyme.

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AAC(6')-Ib     156 SPSNLRAIRCYEKAGFERQGTVTT
AAC(6')-IIa        TPNNHRAIRCYEKAGFVREKIITT
AAC(6')-IIb        SPNNMRAIRCYEKAGFRKVKVVST
AAC(6')-Ie         HKNNPRAIRAYQKSGFRIIEDLPE
AAC(6')-In         ALENTISHAMHRALGFHETERVVY
AAC(6')-Ip2        ALDNTISHAMHRALGFHETERVVY
AAC(6')-Is         AIDNTISHAMHRALGFHETERVVY
AAC(6')-Ir         AIDNTISHAMHQALGFHETERVVY
AAC(6')-Io         TLDNQISHAMHRALGFHETERVVY
AAC(6')-Iq2        ALDNQITHAMHQALGFQETERVVY
AAC(6')-Ih         ALDNQISHAMHQALGFHETERVVY
AAC(6')-Ij         ALDNRISHAMHQALGFHETERVVY
AAC(6')-Im         ALDNTISHAMHRALGFQETECVVY
AAC(6')-Ig         ALDNVISHAMHRSLGFQETEKVVY
AAC(6')-Ik         ALDNVISHAMHRALGFQETERVVY
AAC(6')-If         ALDNHISYQMHQALGFEETERVVF
AAC(6')-Id         STDNPESHRFHQSLGFKETERVVY
AAC(6')-Ic         DIANLDSQRLHAALGFAETERVVF
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FIG. 1. Alignment of amino acid sequences in motif B, one of the conserved regions among AAC(6′) enzymes (11) and other acetyltransferases (8). The top four sequences are of the four members of AAC(6′) subgroup 1, and the sequences of the members of subgroup 2 are outlined by the dotted line. The asterisks indicate the amino acids that are conserved in all of the amino acid sequences of AAC(6′)-I subgroups 1 and 2.

Nucleotide sequence accession number. The nucleotide sequences of the mutants have been deposited in the GenBank sequence library (accession no. AF139864-71).

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