# Purification and characterization of a plasmid-encoded aminoglycoside-(3)-*N*-acetyltransferase IV from *Escherichia coli*

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Plasmid-encoded aminoglycoside-(3)-*N*-acetyltransferase IV, AAC(3)-IV, was purified to homogeneity by affinity chromatography from *E. coli*. The enzyme was shown to consist of a monomer, with the apparent $M_r$ being in agreement with that calculated from the nucleotide sequence of the *aacC4* gene ($M_r$ 28500). Determination of the sequence of the N-terminal 6 amino acids revealed that processing did not occur, indicating the cytoplasmic localization of the AAC(3)-IV enzyme. A correlation of antibiotic resistance with $K_m$ values of the purified enzyme for a corresponding set of aminoglycoside substrates is discussed with respect to the mechanism of resistance in vivo.

*Aminoglycoside-(3)*-N-*acetyltransferase* *Plasmid-encoded gentamicin resistance*

## 1. INTRODUCTION

Resistance to aminoglycoside antibiotics in gram-negative bacteria is most frequently due to the action of plasmid-encoded acetyl- or adenylyltransferases [1]. Some of these aminoglycoside-modifying enzymes have already been purified and characterized [1,2].

Recently, we cloned and sequenced the plasmid pWP7b encoded gene for the aminoglycoside-(3)-*N*-acetyltransferase IV, AAC(3)-IV, enzyme originally isolated from a *Salmonella typhimurium* strain [3]. This enzyme exhibits a broad substrate range, modifying gentamicin, tobramycin, sisomicin, netilmicin, kanamycin, neomycin and the unusual aminoglycoside apramycin [1]. There is a considerable interest for the large-scale purification of an aminocyclitol-modifying enzyme with a broad substrate spectrum, as is exhibited by AAC(3)-IV [1,3], for the quantitative determination of a number of aminoglycoside antibiotics, especially gentamicin and tobramycin, in body fluids [1,4]. Here the structural and kinetic features of the plasmid-encoded AAC(3)-IV were studied in order to give some insight into the mechanism of resistance against aminocyclitol antibiotics in gram-negative bacteria mediated by modifying enzymes.

## 2. MATERIALS AND METHODS

### 2.1. *Plasmids, bacteria and growth conditions*

Plasmids pWP7b, pWP701 and pWP703, and *Escherichia coli* C600 have been described earlier [3]. *E. coli* C600/pWP701 was grown in a Biostat U50D fermenter at 37°C to late log phase in 10-l volumes of a complex medium consisting of 1% tryptone, 0.5% yeast extract, 0.2% glucose, and 1% NaCl, buffered with 10 mM $Na_2HPO_4$, 25 mM $KH_2PO_4$, 25 $\mu$M $CaCl_2$, 250 $\mu$M $MgSO_4$, 3 mM $(NH_4)_2SO_4$, and supplemented with 10 $\mu$g/ml gentamicin.

### 2.2. *Purification of AAC(3)-IV*

All operations were carried out at 4°C. Ribosome-free supernatants (S100) of 80 g *E. coli*

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C600/pWP701 were prepared by disrupting cells by a French pressure cell at 16000 lb/inch$^2$, and centrifugation (1 h, 100000 × *g*) in buffer (20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 30 mM $NH_4Cl$, 6 mM 2-mercaptoethanol). Enriched AAC(3)-IV enzyme was obtained by affinity chromatography with CH-Sepharose to which tobramycin had been covalently coupled as described by the manufacturer (Pharmacia). The S100 was concentrated by precipitation with 65% ammonium sulfate, dialyzed against 10 mM Tris-Cl, pH 7.6 (buffer A), and applied to a 0.7 × 25 cm tobramycin-Sepharose column (flow rate 10 ml/h; fraction size 2.3 ml). After washing with buffer A there was a first elution step with buffer B (100 mM $(NH_4)_2SO_4$ in buffer A) and a second with buffer C (5 ml 20 mg/ml gentamicin in buffer A) and washing with buffer A. Purified AAC(3)-IV enzyme was lyophilized and stored at −20°C. Aminoglycoside acetyltransferase activity and protein concentrations were determined according to [5,6]. Gel electrophoresis was performed as described in [7,8].

2.3. *Characterization of the AAC(3)-IV enzyme*

Partial proteolysis with *Staphylococcus aureus* V8 protease was carried out according to [9], and analysis of the amino acid sequence of the purified AAC(3)-IV enzyme according to the 'DABITC'-method as described in [10]. For the determination of the $M_r$ of the native enzyme, gel filtration using a 2.5 × 120 cm sephacryl S300 column was performed (running buffer: 0.1 M $KH_2PO_4/K_2HPO_4$, pH 7.5; flow rate 23 ml/h).

2.4. *mic values*

Minimal inhibitory concentrations (mic) were determined for *E. coli* C600/pWP7b according to [11] using 0, 1.6, 3.1, 6.2, 12.5, 25, 50, 100, 200 and 400 μg/ml of aminoglycoside antibiotics.

## 3. RESULTS AND DISCUSSION

### 3.1. *Purification of the AAC(3)-IV enzyme*

A typical result of a purification procedure of the AAC(3)-IV enzyme from *E. coli* C600/pWP701 is summarized in table 1. The bulk of the S100 proteins did not bind to the tobramycin-Sepharose column and eluted by washing with buffer A. A minor fraction of the AAC(3)-IV enzyme was eluted with buffer B together with residual non-specifically bound protein, whereas specifically bound aminocyclitol acetyltransferase was removed from the column by buffer C. The quite unusual elution behaviour of the AAC(3)-IV enzyme could probably be attributed to the fact that different primary amino residues of the tobramycin molecule had been covalently coupled to the Sepharose matrix thus resulting in heterogenous binding affinities of the AAC(3)-IV enzyme. Moreover, aminoglycoside Sepharose was described to act as anion exchange material because of the polycationic nature of these antibiotics [1]. However, the AAC(3)-IV enzyme, which was found to comprise at least 1% of the S100 proteins (fig.1A) was enriched 63-fold by affinity chromatography (table 1).

One-dimensional SDS-gel electrophoresis revealed that the purified enzyme consisted of two molecular species with apparent $M_r$ of 27500 and 29500, respectively (fig.1B); no other protein species were detectable. Two-dimensional gel electrophoresis [8] of the purified enzyme resulted in

Table 1

Purification of the AAC(3)-IV enzyme

| Step | Total protein (mg) | Total activity (μkat) | Specific activity (μkat/mg protein) | Recovery (%) | Purification (-fold) |
|---|---|---|---|---|---|
| 1. S100 | 2700 | 87 | 0.032 | 100 | 1 |
| 2. Tobramycin-Sepharose, elution with $(NH_4)_2SO_4$ | 390 | 39 | 0.1 | 45 | 3.1 |
| 3. Tobramycin-Sepharose, elution with gentamicin | 6 | 12 | 2.0 | 14 | 63 |

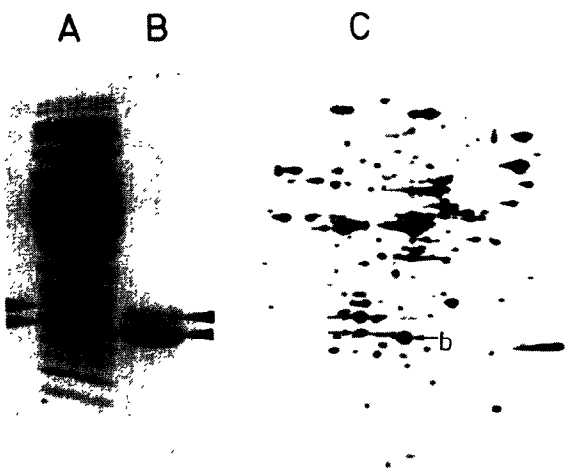


Fig.1. Gel electrophoretic mobility of the AAC(3)-IV enzyme. One-dimensional SDS-polyacrylamide gel electrophoresis [7] of (A) S100 of *E. coli* C600/pWP701, and (B) purified AAC(3)-IV enzyme. (C) Two-dimensional gel electrophoresis of a total cell extract of strain *E. coli* C600/pWP703 according to O'Farrell [8]. Arrows indicate the two polypeptide forms of the enzyme; b, mature form of $\beta$-lactamase [3].

two spots (not shown) with identical isoelectric points and $M_r$ values corresponding to those observed in one-dimensional gels. Separation of an extract of *E. coli* C600/pWP703 (fig.1C) by the same system allowed estimation of the isoelectric points to be about 5.6 when other *E. coli* proteins identified on O'Farrell gels [12] or the slightly more acidic TEM-1 $\beta$-lactamase [13] were used as internal standards.

There are a number of possible explanations for the occurrence of two bands in SDS-gel electrophoresis: (i) Enrichment of two different cellular proteins by affinity chromatography, one of which exhibits aminocyclitol acetyltransferase activity, (ii) the AAC(3)-IV enzyme consists of two subunits, (iii) processing or modification of a precursor, (iv) partial degradation by proteases, (v) premature termination or partial 'read-through' through a stop-codon, (vi) alternate usage of a different reading frame, or (vii) different migration behaviour of two stable conformations of the AAC(3)-IV enzyme in the presence of SDS. The following findings support the last mentioned explanation: (i) When either of the two bands in SDS gels were rerun with the same method again a separation into two bands occurred. (ii) Expression of plasmid pWP701 in minicells [3] resulted in the same products as were observed by gel electrophoresis of the purified AAC(3)-IV enzyme. (iii) DNA sequence analysis of the *aacC4* gene [3,11] revealed that there is only one open reading frame coding for a protein with $M_r$ 28500, which is in good agreement with the size of the two products of the AAC(3)-IV enzyme determined by gel electrophoresis. The derived amino acid sequence for the acetyltransferase does not contain a typical procaryotic signal sequence [14]. The sequence of the N-terminal 6 amino acids of the AAC(3)-IV enzyme was determined to be ($NH_2$)–Met-Gln-Tyr-Glu-Trp-Arg...–(COOH), which corresponds to the one proposed from the DNA sequence for the *aacC4* gene [11]. N-terminal processing did not occur except for the removal of the formyl residues from methionine which resulted in covalent coupling of the protein to isothiocyanate compounds. (iv) Partial proteolysis with *S. aureus* V8 protease yielded the same pattern of peptides for the two proteins when isolated from SDS gels (not shown). These results agree with the suggested two conformational forms of the AAC(3)-IV enzyme in polyacrylamide gels. Nevertheless, for a final conclusion further data disproving other explanations such as C-terminal processing are required.

In the past, there has been discussion on the localization to the periplasmic space of aminoglycoside modifying enzymes in gram-negative bacteria [1]. Recently, the presence of an amikacin phosphorylating enzyme, APH(6′), in the cytoplasm was shown [15]. The absence of a signal sequence and of N-terminal processing indicated that the AAC(3)-IV enzyme was similarly localized in the cytoplasm. Also, there is no evidence for a characteristic signal sequence [14] usually found at the N-terminus of excreted proteins for any of the already sequenced aminoglycoside resistance genes [11,17]. Taken together, these results support a cytoplasmic localization of aminocyclitol-modifying enzymes in general.

### 3.2. *Structural and kinetic features of the AAC(3)-IV enzyme*

Gel filtration showed that the purified AAC(3)-IV enzyme was monomeric under the tested condition (not shown; see section 2). Acetyltransferase

Table 2

$K_m$ values for the AAC(3)-IV enzyme

| Antibiotics | $K_m$ values for purified AAC(3)-IV ($\mu$M) |
|---|---|
| Gentamicin (complex) | 177 |
| Gentamicin C1 | 87 |
| Gentamicin C1a | 195 |
| Gentamicin C2 | 142 |
| Sisomicin | 174 |
| Kanamycin | 241 |
| Tobramycin | 26 |
| Apramycin | 11 |

activity was dependent neither on the pH in the range pH 7–8.5 nor on the presence of divalent cations ($Mg^{2+}$), though the activity was optimal at 0.2 mM $MgCl_2$.

The $K_m$ values for the AAC(3)-IV enzyme using several aminoglycoside substrates are listed in table 2, and are in good correlation with the $K_m$ values for similar enzymes [1]. A correlation of antibiotic resistance with the $V_{max}/K_m$ ratio of enzymatic modification of aminoglycosides by a kanamycin acetyltransferase, AAC(6′)-IV, was proposed [15]. The mic values of strain *E. coli* C600/pWP7b for gentamicin C-complex (50 $\mu$g/ml), sisomicin (50 $\mu$g/ml), kanamycin (3.1 $\mu$g/ml), tobramycin (50 $\mu$g/ml), and apramycin (200 $\mu$g/ml) do not strictly correlate with the kinetic properties of the enzyme (see table 2), though the best substrate, apramycin, also shows the highest resistance level. This could be due to several other factors contributing to the level of resistance against aminoglycosides in a bacterial strain containing a plasmid-encoded modifying enzyme, such as uptake rates or the affinity of the ribosomes to the antibiotics.

The purified AAC(3)-IV enzyme could be used for affinity labeling with UV activatable aminocyclitol compounds [16] for the identification of amino acid residues being involved in the binding of substrates to the catalytic center of the enzyme.

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