Biophysical and enzymatic properties of aminoglycoside adenylyltransferase AadA6 from *Pseudomonas aeruginosa*

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**A B S T R A C T**

The gene coding for the aminoglycoside adenylyltransferase (aadA6) from a clinical isolate of *Pseudomonas aeruginosa* was cloned and expressed in *Escherichia coli* strain BL21(DE3)pLysS. The overexpressed enzyme (AadA6, 281 amino-acid residues) and a carboxy-terminal truncated variant molecule ([1-264]AadA6) were puriﬁed to near homogeneity and characterized. Light scattering experiments conducted under low ionic strength supported equilibrium between monomeric and homodimeric arrangements of the enzyme subunits. Circular Dichroism spectropolarimetry indicated a close structural relation to adenylyl kinases. Both forms modiﬁed covalently the aminoglycosides streptomycin and spectinomycin. The enzyme required at least 5 mM MgCl2 for normal Michaelis–Menten kinetics. Streptomycin exhibited a strong substrate inhibition effect at 1 mM MgCl2. The truncated 17 residues at the C-terminus have little influence on protein folding, whereas they have a positive effect on the enzymic activity and stabilize dimers at high protein concentrations (> 100 μM). Homology modelling and docking based on known crystal structures yielded models of the central ternary complex of monomeric AadA6 with ATP and streptomycin or spectinomycin.

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1. Introduction

Antibiotic resistance of pathogenic bacteria has received immense attention over the last years as the rapid increase of multiresistant bacterial strains along with the lack of new antibiotics became a threat to global public health [1–3]. Aminoglycosides are a relatively large class of antibiotics that have been employed extensively over the past decades against aerobic bacterial pathogens [4,5]. They target accessible regions of polyanionic 16S rRNA on the 30S ribosomal subunit, notably the A site for aminocyl-tRNA binding [6]. Following the discovery of streptomycin [7] in 1944 aminoglycosides have been widely used due to their bactericidal action and their observed synergy with other antibiotics [8]. Against this class of compounds the bacteria elaborated three mechanisms of resistance: decreased permeability of the cell wall [9], ribosome alteration [10] and enzymatic modiﬁcation [11]. The latter mechanism is of most clinical importance as it confers high level of resistance and the genes encoding aminoglycoside modifying enzymes can be disseminated by plasmids or transposons [12]. Aminoglycoside modifying enzymes catalyze the modiﬁcation of hydroxyl or amino groups of 2-deoxystreptamine or the sugar moieties and can be acetyltransferases (AACs), nucleotidytranferases (ANTs), or phosphotransferases (APHs) [13]. Evolution by mutagenesis of the genes encoding the aminoglycoside modifying enzymes led to a high number of variants that can utilize an ever growing number of aminoglycoside analogues as substrates. The investigation of the function of such enzymes is expected to drive the production of efﬁcient inhibitors that can be used in combination with the known antibiotics to reduce signiﬁcantly infections by resistant pathogens. A streptomycin/spectinomycin adenylyltransferase gene (aadA) was ﬁrst identiﬁed and sequenced [14] in *E. coli* and recently the crystal structure of an aminoglycoside adenylyltransferase (AadA) from *Salmonella enterica* has been determined at 2.5 Å resolution [15]. Herein, we
investigated the protein encoded by the gene aadA6 identified in a clinical multiresistant isolate of *P. aeruginosa* Ps100 located in the integron In118 [16,17]. The protein termed AadA6 belongs to the subclass of 3′-O-nucleotidyltransferases (ANT3′), it consists of 281 amino-acid residues (UniProt: Q9RG2C) and confers resistance to streptomycin and spectinomycin [16]. The sequence information collected from the Conserved Domain Database (CDD) [18] for this protein points to the conserved nucleotidyltransferase (NTase) domain lying between residues 10 and 110. This domain includes the nucleotide triphosphate binding site and one Mg2+ binding site [19,20].

In the present work circular dichroism spectra, laser light scattering and kinetic data have been measured for purified AadA6 and a C-terminal truncated variant of the enzyme. The latter was termed [1-264]AadA6 and was produced in an attempt to achieve an enzyme form easier to crystallize than the wild type. Finally, two theoretical models of the structures of the AadA6 ternary complexes with ATP and streptomycin or spectinomycin were built based on the homologous AaDA from *S. enterica* [15].

2. Materials and methods

2.1. Gene cloning

Total DNA from the *P. aeruginosa* strain Ps100 was purified by QIAamp DNA mini kit (Qiagen, Germany) and used in order to amplify the coding sequence of the aadA6 gene by PCR. The primers were designed according to sequence information obtained by direct sequencing of the aadA6 gene containing integron of the *P. aeruginosa* strain Ps100 as described previously [21] (GenBank: AV460081). The PCR products were purified by the Jetquick kit (Genomed, Germany) were digested with the Ndel/BamHI endonucleases and ligated in the corresponding restriction sites of the pT7-7 (ampR) expression vector. The recombinant plasmids were transformed in *E. coli* BL21(DE3) pLysS cells. Transformed bacteria were grown in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The site of insertion of the PCR products was confirmed by Sanger sequencing of the purified recombinant plasmids (Macrogen, Europe).

2.2. Protein expression and purification

Four liters of LB medium containing 1 mM MgCl2 were inoculated with 40 ml of an overnight culture of each of the recombinant strains and let grow under shaking at 220 rpm (37 °C). When the absorbance at 600 nm reached 0.5–0.8 the temperature was decreased to 18 °C and growth continued for 16 more hours in the presence of 0.3 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). 16 g of cell paste was resuspended in 16 ml lysis buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl) containing 4 mM mercaptoethanol and 2 mM MgCl2. Lysozyme was added to 0.3 mg/ml and the supernatant was taken for phosphate determination. The antibiotic substrates spectinomycin and dihydrostreptomycin were added to 20–120 and 20–160 μM when spectinomycin and dihydrostreptomycin were analysed, respectively. Pre-incubation of all reaction components except the analysed enzyme was performed for 10 min. The latter procedure allowed the consumption of probable endogenous phosphates so that they did not interfere with the measurements of enzyme activity. All kinetic data were collected in triplicates. Oxidized streptomycin was also tested though in a limited number of reactions and showed the same kinetic behaviour with its reduced counterpart.

2.3. In vitro enzymatic activity

Steady-state enzyme kinetics monitored the phosphate as end-product of the adenyltransferase action combined with that of inorganic pyrophosphate. The analytical method employed was a modification of the colorimetric method based on the reaction of malachite green, molybdates and phosphates [22,23]. The initial rates were measured in reaction mixtures of 65 nM enzyme and varying substrate concentrations in 50 mM Tris–HCl pH 7.5 buffer containing 15 mM MgCl2 at 23 °C. In the earlier experiments 1, 5 and 10 mM MgCl2 were also present during the kinetic analyses. Each reaction was stopped at the selected time intervals by addition of 26 μl 2.25N H2SO4. After standing for 10 min at 0 °C, the mixture was centrifuged for 3 min in order to remove the protein and the supernatant was taken for phosphate determination. The antibiotic substrates spectinomycin and dihydrostreptomycin varied from 2–20 μM. ATP varied from 20–120 and 20–160 μM when spectinomycin and dihydrostreptomycin were analysed, respectively. Pre-incubation of all reaction components except the analysed enzyme was performed for 10 min. The latter procedure allowed the consumption of probable endogenous phosphates so that they did not interfere with the measurements of enzyme activity. All kinetic data were collected in triplicates. Oxidized streptomycin was also tested though in a limited number of reactions and showed the same kinetic behaviour with its reduced counterpart.

2.4. Data analysis

Based on earlier results for a closely related enzyme [24] the same steady-state assumption was made for sequential ordered bi-substrate reactions. In this case, Eq. (1) provides the initial velocities and was fitted with the measured initial rates when both substrates were varied. Non-linear fits of the data to Eq. (1) were carried out using Graft 7.0 (Erithacus Software Ltd., Horley, U.K.).

\[
\nu = V_{\text{max}}[A][B]/(K_{m_A}K_{m_B} + K_{m_A}[A] + K_{m_B}[B] + [A][B])
\]  

(1)

In the above equation, \(\nu\) is the initial velocity, \(V_{\text{max}}\) is the maximal velocity, \(K_{m_A}\) and \(K_{m_B}\) are the Michaelis–Menten constants of substrates A and B, respectively and \(K_D\) is the dissociation constant of the first substrate A that is added to the enzyme. In the present analysis, A and B refer to ATP and streptomycin or spectinomycin, respectively.

2.5. Biophysical characterization

Circular Dichroism spectropolarimetry measurements of the two forms of the purified enzyme were conducted with a J-810 spectropolarimeter (JASCO, Inc., USA). The data were processed using both software and reference databases of the DichroWeb server [25]. For the circular dichroism spectra protein solutions of 0.2 mg/ml were used in 50 mM potassium phosphate buffer pH 7.5 whereas for the thermal unfolding experiments solutions of 0.1 mg/ml were prepared in the same buffer. During the melting experiments the ellipticities (θ) at wavelength 209 nm corresponding to the minimum value of the spectra, were recorded with increasing temperature. CD data for the full-length AadA6 and truncated enzyme [1-264]AadA6 have been deposited in the Protein Circular Dichroism Data Bank (PCDDB) [26] with PCDDB id codes: CD0004574000 and CD0004575000, respectively.

The dynamic light scattering [27] of samples containing approximately 4 mg/ml of enzyme were analysed in 50 mM Tris–HCl pH 7.5 buffer solutions with two concentrations of MgCl2 (1 and 18 mM for AadA6, 1 and 15 mM for [1-264]AadA6) at 20 °C with the HELEOS8 MALS (Multi-angle light scattering) instrument
(Wyatt Technology Corp., Santa Barbara, CA, USA). The instrument operated in combination with a Superdex 200HR 10/30 pre-packed gel filtration column (GE Healthcare). The light scattering experiments were carried out also in the presence of 2 mM of both AMPCPP and streptomycin. The quasi-elastic scattering data along with the refractive index and UV absorbance measurements were analysed with software ASTRA® 6.1.117. This analysis allowed also the estimation of the molecular masses of the eluates and thus the assignment of the correct oligomeric state of the protein.

2.6. Homology modelling

A model for the structure of AadA6 (3-262) was obtained from the Swiss-Model protein server [28] based on the homologous AadA (identity ~45%) from S. enterica (PDB: 4CS6), which served as template. For this model, a structural alignment of the N-terminal domain of AadA with chain A of kanamycin nucleotidyltransferase (KNTase) (PDB: 1KNY) was generated, overlapping mainly the β-strands in this domain. The aim of the latter alignment was to locate the ATP and aminoglycoside binding sites. The structural alignment showed that there are certain characteristic substrate binding residues in AadA like in KNTase. This allowed the placement of the substrates ATP and streptomycin to the corresponding binding sites in KNTase. Finally, the model was energy minimized with the program CNS [29] using coordinates and modified geometrical restraint files from the HIC-Up database (xray.bmc.uu.se/hicup, [30]). The coordinates of the derived AadA6 theoretical model are available at the Model Archive (www.modellarchive.org) under accession number MA-AWT7Q. In addition, a second model of AadA6 was derived with ATP and spectinomycin in the enzyme’s active site following analogous procedures as described above.

3. Results

3.1. Strains and antibiotic profile

Clinical strain Ps100 was highly resistant against streptomycin, gentamycin and amikacin [MIC (Minimum Inhibitory Concentration) > 256 μg/ml], while the recombinant E. coli strains expressing either the full length or the truncated AadA6 exhibited resistance against streptomycin but not gentamycin or amikacin, indicating that the resistant phenotype was conferred by the corresponding determinant. The MIC values for streptomycin were assigned the observed differences to the distinct expression levels of AadA6 for spectinomycin comparing to the highly homologous enzyme from S. marcescens.

Moreover, kanamycin A, gentamycin, and amikacin were also tested as substrates for AadA6 with no detectable rates of reaction. Kanamycin A was actually found to be competitive inhibitor for the aminoglycoside substrates.

Finally, it is worth noting that when the initial velocity measurements were carried out in the presence of 1 mM MgCl2 a strong substrate inhibition effect was invariably observed by streptomycin. This was largely alleviated by increasing the concentration of MgCl2 to 5 mM. An analogous Mg2+ concentration effect was also observed in the case of kanamycin nucleotidyltransferase [32] as well as other aminoglycoside adenyltransferases [33].

3.4. Biophysical characterization

Analysis of the far-UV (190–240 nm) CD-spectra shown in Fig. 1A provided for AadA6 58% α-helical and 19% β-sheet content. The respective values for [1-264]AadA6 were 52% and 23%. The CD spectra of AadA6 revealed remarkable similarity to that of adenylate kinase (PDB: 3ADK), which is used in various reference data sets for the estimation of secondary structure by CD spectroscopy.

In order to probe the thermal denaturation of both forms of the enzyme, CD measurements were conducted as function of temperature (Fig. 1B). The melting temperatures (Tm) for AadA6 and [1-264]AadA6 were estimated by interpolation on the melting curves as 49.5 and 51.0 ± 0.5 °C, respectively. The thermal unfoldings of the full-length and truncated enzymes were found to occur in one irreversible step.

In addition, laser light scattering experiments were carried out for both AadA6 and [1-264]AadA6 in combination with an analytical gel filtration column. The results of two selected experiments are shown in Fig. 2 and a complete list of the derived molecular hydrodynamic radii is given in Table 2.

Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kcat (s⁻¹)</th>
<th>Km (μM)</th>
<th>kcat/Km (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AadA6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>streptomycin</td>
<td>0.66 ± 0.06</td>
<td>1.8 ± 0.6</td>
<td>(3.7 ± 1.3) × 10³</td>
</tr>
<tr>
<td>spectinomycin</td>
<td>0.63 ± 0.03</td>
<td>0.4 ± 0.1</td>
<td>(1.6 ± 0.4) × 10³</td>
</tr>
<tr>
<td>ATP</td>
<td>0.61 ± 0.03</td>
<td>11.0 ± 1.2</td>
<td>(5.5 ± 0.7) × 10⁴</td>
</tr>
<tr>
<td>[1-264]AadA6 streptomycin</td>
<td>0.47 ± 0.04</td>
<td>3.3 ± 0.7</td>
<td>(1.4 ± 0.3) × 10⁴</td>
</tr>
<tr>
<td>spectinomycin</td>
<td>0.28 ± 0.02</td>
<td>0.2 ± 0.1</td>
<td>(1.3 ± 0.7) × 10⁶</td>
</tr>
<tr>
<td>ATP</td>
<td>0.41 ± 0.02</td>
<td>10.5 ± 0.8</td>
<td>(3.9 ± 0.4) × 10⁴</td>
</tr>
</tbody>
</table>

AadA6 exhibits high degree of sequence identity (264/268 residues) to the nucleotidyltransferase domain [ANT(3’)-I] of the bifunctional, nucleotidyl- and acetyl-transferase aminoglycoside modifying enzyme from Serratia marcescens (UniProt: Q8VQN7), which has been extensively characterized with regard to its enzymatic properties [24]. The high homology between the enzymes suggests that AadA6 acts in a similar fashion. We decided to purify the truncated form of AadA6 based on the length of the above mentioned closely related domain of the bifunctional enzyme and on the results of sequence comparisons with other homologous proteins [31] using the Universal Protein Resource Knowledgebase. Enzymatic assays of product inorganic pyrophosphate using streptomycin as substrate provided specific activities of 1.3 ± 0.1 and 0.9 ± 0.1 μmol min⁻¹ mg⁻¹ for homogeneous samples of AadA6 and [1-264]AadA6, respectively. The latter values indicate that the truncated enzyme is about 70% as active as the original full-length enzyme in vitro. The observed decrease of activity is due to the truncation of the 17C-terminal residues. The active site comprises mainly N-terminal residues according to the theoretical models described below (Section 3.5). The determined kinetic parameters for AadA6 are summarized in Table 1. These results agree to the earlier reported values for the bifunctional enzyme [24] except of the Km value for spectinomycin, which was calculated to be about four times lower. This leads to a five-fold higher specificity of AadA6 for spectinomycin comparing to the highly homologous enzyme from S. marcescens.
dimeric and monomeric species of AadA6, with mean hydrodynamic radii of 3.4 ± 0.1 and 2.2 ± 0.2 nm, respectively. The same holds true for [1-264]AadA6 with respective values 3.3 ± 0.1 and 2.4 ± 0.1 nm. Addition of MgCl2 and substrate or substrate analogues led to a decrease of the apparent size of the proteins. The latter effect was more prominent for the monomeric form of AadA6. MALS data collected from solutions with ionic strength values of 60–150 mM showed that AadA6 is in equilibrium with an approximate 1:1 dimer to monomer ratio whereas the corresponding value for [1-264]AadA6 was about 1:4. This latter observation indicates that the deleted C-terminal residues favour the formation of protein dimers at concentrations approximately 100 μM used in these light scattering experiments. The occurrence of monomers is in agreement with the recently reported structure of AadA from S. enterica [15].

Moreover, the ionic strength seems to influence the dimerization of the molecule. Light scattering experiments of ~400 μM [1-264]AadA6 in high ionic strength solutions (~350 mM) showed that the protein is dimeric (data not shown). This finding is in agreement with the reported structures of the distantly related (identity < 20%) kanamycin nucleotidyltransferase [20] from S. aureus (PDB: 1KNY) and aminoglycoside 4'-O-adenylyltransferase [ANT(4'-O-AMPCPP) and kanamycina Dimer Monomer]

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>AadA6</th>
<th>[1-264]AadA6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl2 0.1</td>
<td>3.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>MgCl2 1.9</td>
<td>3.3</td>
<td>3.0</td>
</tr>
<tr>
<td>MgCl2 3.2</td>
<td>2.9</td>
<td>2.4</td>
</tr>
<tr>
<td>MgCl2 3.3</td>
<td>2.5</td>
<td>2.4</td>
</tr>
<tr>
<td>MgCl2 3.4</td>
<td>2.4</td>
<td>2.2</td>
</tr>
</tbody>
</table>

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The enzyme kinetic experiments presented in this work for AadA6 from P. aeruginosa and its truncated variant [1-264]AadA6 revealed clear and measurable deviation of the catalytic activity between the two forms. AadA6 was found to be about 40% more active than [1-264]AadA6 in modifying streptomycin indicating that the 17 residues at the C-terminus, which are missing in [1-264]AadA6 are necessary for full activity. The same 17C-terminal residues also support protein dimerization at high enzyme concentrations (> 100 μM) as is derived from MALS experiments contacted under ionic strength conditions (60–150 and 350 mM) close to the physiological values (> 100 mM) [34]. Moreover, AadA6 exhibits higher specificity for spectinomycin, which is retained in the truncated form. The higher specificity for spectinomycin compared to streptomycin may be explained theoretically as follows. The model derived for the structure of the complex of AadA6 with ATP and spectinomycin shows five H-bonds between the enzyme and the antibiotic whereas the respective H-bonds with streptomycin are only three. This leads to a better binding of spectinomycin compared with streptomycin and thus higher specificity for spectinomycin. In the presence of 1 mM MgCl2 used in the early experiments a prominent substrate inhibition was observed with streptomycin for concentrations greater than 3 μM. It is noteworthy that this MgCl2 concentration lies close to the reported physiological values of uncomplexed, free magnesium ions (0.9–1.5 mM) for the S. enterica [35]. Substrate inhibition as well as the decrease of the protein size observed with the MALS experiments at a low enzyme concentration (65 nM), which justifies for the exclusive presence of the monomeric form. A different arrangement of the substrates may be possible on two cooperating active sites in case the enzyme is to function as dimer.

Sequence comparisons suggest that AadA6 belongs to the superfamily of ancient nucleotidytransferases involved in diverse biological functions that range from DNA repair to regulation of biosynthetic pathways and antibiotic resistance [36]. Detailed structure comparisons with members of this superfamily have to wait for the determination of the three-dimensional structure of AadA6 under various conditions.

Finally, the herein described biochemical and biophysical properties along with the supplied structural models provide insight for substrate binding with AadA6 and may facilitate future attempts to design inhibitors that will allow the re-usage of the first generation antibiotics like the aminoglycosides against multiresistant pathogens.

5. Note

This paper is dedicated to the memory of our late colleague Dr. Yannis Papanikolau who had generously advised us on the protein purification procedures.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.09.011.
References