

Communication

Spermidine Binding to the *Acinetobacter baumannii* Efflux Protein AceI Observed by Near-UV Synchrotron Radiation Circular Dichroism Spectroscopy

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Abstract: The aim of this work was to test polyamines as potential natural substrates of the *Acinetobacter baumannii* chlorhexidine efflux protein AceI using near-UV synchrotron radiation circular dichroism (SRCD) spectroscopy. The Gram-negative bacterium *A. baumannii* is a leading cause of hospital-acquired infections and an important foodborne pathogen. *A. baumannii* strains are becoming increasingly resistant to antimicrobial agents, including the synthetic antiseptic chlorhexidine. AceI was the founding member of the recently recognised PACE family of bacterial multidrug efflux proteins. Using the plasmid construct pTTQ18-aceI(His₆) containing the *A. baumannii* aceI gene directly upstream from a His₆-tag coding sequence, expression of AceI(His₆) was amplified in *E. coli* BL21(DE3) cells. Near-UV (250-340 nm) SRCD measurements were performed on detergent-solubilised and purified AceI(His₆) at 20 °C. Sample and SRCD experimental conditions were identified that detected binding of the triamine spermidine to AceI(His₆). In a titration with spermidine (0-10 mM) this binding was saturable and fitting of the curve for the change in signal intensity produced an apparent binding affinity (K_D) of 3.97 +/- 0.45 mM. These SRCD results were the first experimental evidence obtained for polyamines as natural substrates of PACE proteins.

Keywords: *Acinetobacter baumannii*; antimicrobial resistance; circular dichroism spectroscopy; efflux protein; efflux pump; foodborne pathogen; hospital-acquired infection; polyamine

1. Introduction

The Gram-negative bacterium *Acinetobacter baumannii* has become a highly successful pathogen and a major cause of hospital-acquired infections [1]. *A. baumannii* is also an important foodborne pathogen [2] and strains of *A. baumannii* are becoming increasingly resistant to antimicrobial agents, including last-resort antibiotics such as carbapenems [3]. Consequently, *A. baumannii* is on the World Health Organisation (WHO) “priority pathogens” list for research and development of new antibiotics (<https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed>) as “Priority 1: CRITICAL”. The *A. baumannii* gene A1S_2063 (<https://www.ncbi.nlm.nih.gov/gene/4919307>) was found to be upregulated in response to the bisbiguanide antiseptic chlorhexidine (1,6-bis(4-chloro-phenyl)-biguanido)hexane through transcriptomic analysis. This gene, later renamed *aceI* (<https://www.ncbi.nlm.nih.gov/gene/66396841>), was shown to code for a chlorhexidine efflux protein through sequence and biochemical analysis [4].

The AceI protein (*Acinetobacter* chlorhexidine efflux protein I) (<https://www.uniprot.org/uniprot/P0DUT9>) was the founding member of the PACE (proteobacterial antimicrobial compound efflux) family of bacterial multidrug efflux proteins [5,6]. PACE proteins contain ~150 amino acids and sequence analysis suggests

that they form four transmembrane-spanning α -helices arranged as two tandem bacterial transmembrane pair (BTP) domains [7]. The relatively small size of PACE proteins means that they are likely to function as an oligomer. A mass spectrometry study demonstrated that AceI exists in a monomer-dimer equilibrium in solution, where AceI forms dimers at high pH and binding to chlorhexidine facilitates the functional form of the protein [8]. A high-resolution three-dimensional structure of a PACE protein is yet to be elucidated.

Because chlorhexidine is a synthetic biocide used only since the 1950s, chlorhexidine efflux will not be the physiological function of AceI, so the resistance to chlorhexidine that PACE proteins provide is fortuitous. Possible natural substrates of AceI that we investigated included polyamines such as spermidine, spermine, putrescine and cadaverine that have some chemical and structural similarity to chlorhexidine. Polyamines are primordial compounds found abundantly in eukaryotes, bacteria and archaea [9], and they have multiple roles in bacterial pathogens [10,11]. Polyamines may exist at high (millimolar) concentrations in cells and they can inhibit cell growth when they are in excess, so it makes sense for cells to have detoxification mechanisms for polyamines such as active efflux.

One of the first types of biophysical experimental evidence that we obtained for chlorhexidine binding to AceI was done through near-UV synchrotron radiation circular dichroism (SRCD) spectroscopy using methods developed with and applied to other membrane proteins [12-16]. By this method chlorhexidine binding to detergent-solubilised and purified AceI had an apparent binding affinity (K_D) of 5.8 μ M [4]. We therefore used near-UV SRCD spectroscopy to test for polyamines binding to AceI and here we report the first experimental evidence obtained for a polyamine binding to a PACE protein.

2. Materials and Methods

2.1. Protein Expression and Purification

The *A1S_2063/aceI* gene was previously introduced into expression plasmid pTTQ18 immediately upstream of a His₆-coding sequence [4]. For producing sufficient quantities of purified AceI(His₆), *E. coli* BL21(DE3) cells carrying plasmid pTTQ18-aceI(His₆) were grown in Luria-Bertani broth containing ampicillin (100 μ g/mL) in a 30-L fermentor and AceI(His₆) expression was induced with isopropyl- β -D-galactopyranoside (IPTG) [4]. Cells were harvested, resuspended in 20 Tris-HCl (20 mM, pH 7.6), EDTA (0.5 mM), and glycerol (10% v/v) and stored at -80 °C. At a later time, cells were thawed at 4 °C and disrupted using a Constant Systems cell disruptor. Inner membranes were isolated by sucrose density gradient ultracentrifugation, snap frozen in Tris-HCl (20 mM, pH 7.6) and stored at -80 °C. Membranes were then solubilized in the mild detergent *n*-dodecyl- β -D-maltoside (DDM) and purified by Ni-NTA affinity chromatography [17].

2.2. Near-UV SRCD Spectroscopy

Measurements were performed using a nitrogen flushed CD instrument on Beamline B23 at the Diamond Light Source Ltd (Oxfordshire, UK). Samples contained purified AceI(His₆) (20 μ M or 40 μ M) in potassium phosphate buffer (10 mM, pH 7.6) plus 5% glycerol and 0.05% DDM. The sample cell had a path length of 1 cm and aperture 2 mm (minimum volume 60 μ L) and used a 1 mm collimated beam. Spectra in the wavelength range 250-340 nm were obtained at 20 °C using slit widths of 1 mm, increment 0.5 nm and integration time of 1 s. All spectra were an average of 10 scans acquired over approximately 1 h. Spectra were zeroed at a wavelength of 335 nm and corrected for any signals coming from buffer or from the added polyamines themselves by subtraction of the relevant spectra acquired on these sample components alone, as appropriate. Spectra are presented in units of mean residue ellipticity ($[\theta]$ MRE, deg.cm².dmol⁻¹) and all measurements had PMT values below 600 V. For quantifying spermidine binding, the polyamine was titrated with AceI up to a concentration of 10 mM. At each titration point, the sample cuvette was removed from the instrument, an appropriate volume of a higher

concentration spermidine solution was added, followed by gentle mixing with a pipette before returning to the instrument and recording the next spectrum. The change in $[\theta]_{\text{MRE}}$ at each titration point was measured at six different wavelengths in the region 255-260 nm. Average values for the change in $[\theta]_{\text{MRE}}$ were fitted to the Michaelis-Menten equation using GraphPad Prism 6 software to produce the apparent dissociation constant. Stability measurements confirmed that there was no significant change in spectra of the protein alone when continuously measured over a period of 12 h, which is longer than that of an entire titration with ligand.

3. Results and Discussion

Using a trial and error approach, sample and near-UV SRCD experimental conditions were identified and optimised for observing the triamine spermidine binding to DDM-solubilised and purified AceI(His₆). At concentrations of 20 μM and 40 μM , AceI(His₆) the binding of 5 mM spermidine was detected in spectra recorded in the near-UV region (250-340 nm), with the most prominent effects on the spectra at 255-295 nm. Under the same conditions, binding of the longer-chain tetraamine spermine was not detected (Figure 1). In order to quantify the binding of spermidine a titration of 40 μM AceI(His₆) with spermidine up to 10 mM was performed (Figure 1). Stability measurements confirmed that there was no significant change in spectra of the protein alone when continuously measured over a period of 12 h, which is longer than the entire titration with ligand. A saturable change in signal intensity on titration with spermidine was measured in the region 255-260 nm, which corresponds to the wavelength range for phenylalanine residues. Fitting a mean of the change in signal intensity in this region gave an apparent binding affinity (K_D) of 3.97 \pm 0.45 mM (Figure 2). Whilst the binding of spermidine to AceI has a three-fold lower affinity than the binding of chlorhexidine, a lower binding affinity for a natural substrate of a transporter is expected. The SRCD results presented here were the first experimental evidence obtained for a polyamine binding to a PACE protein.

4. Conclusions

The results presented in this paper initiated further study on polyamines as potential natural substrates of AceI and other PACE proteins. Future work on the relatively uncharacterised PACE family should include gene cloning, recombinant expression, purification, reconstitution and stability screening of further PACE proteins for application of a multitude of chemical, biochemical, biophysical and computational techniques to elucidate high-resolution structures and to investigate their oligomeric state, physiological function, ligand interactions and substrate specificity, molecular mechanism and dynamics. This may provide information to assist the development of agents or strategies to block the biocide efflux function of PACE proteins.

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Conflicts of Interest: There are no conflicts of interest to declare.

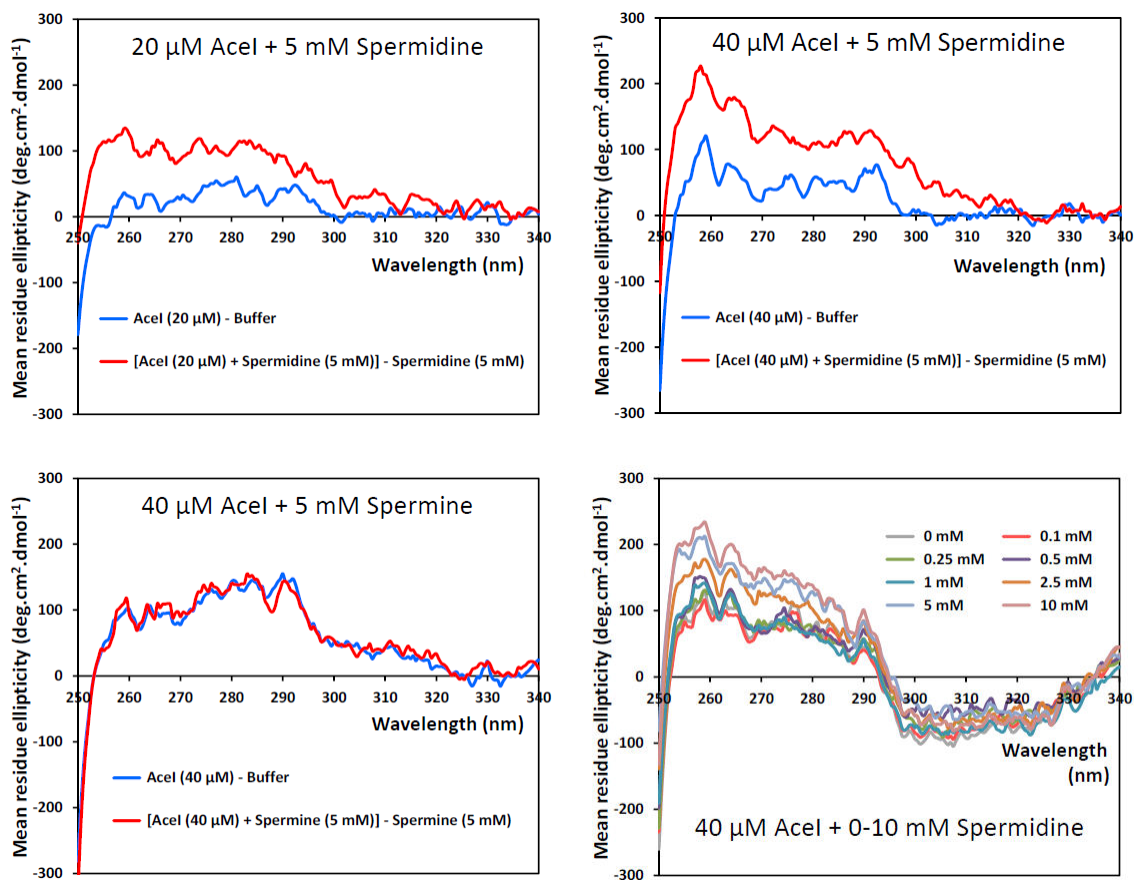


Figure 1. Spermidine binding to the *A. burmannii* efflux protein Acel observed by near-UV synchrotron radiation circular dichroism spectroscopy. Near-UV SRCD spectra (250–340 nm) at 20 °C for samples of DDM-solubilised Acel(His₆) (20 μM or 40 μM in 10 mM KPi pH 7.6, 5% glycerol, 0.05% DDM) showing the effect of spermidine (5 mM) (top) or spermine (5 mM) (bottom left) and a titration with spermidine (0–10 mM) (bottom right). All spectra are an average of 10 scans acquired over approximately 1 h. Spectra were corrected for any signals coming from buffer or from the polyamines themselves by subtraction of the relevant spectra acquired on these sample components alone, as appropriate.

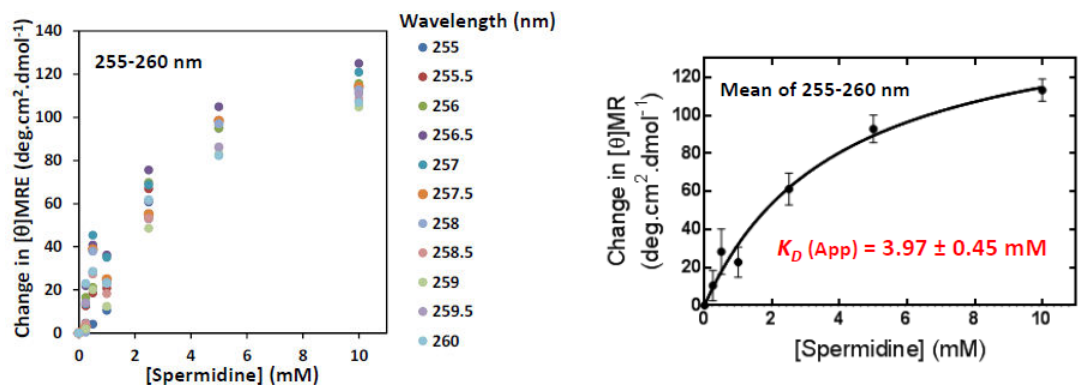


Figure 2. Quantification of spermidine binding to the *A. burmannii* efflux protein Acel by near-UV synchrotron radiation circular dichroism spectroscopy. From the spectra showing the titration of Acel(His₆) with spermidine (Figure 1, bottom right), the change in mean residue ellipticity ([θ]MRE) at each titration point was measured at six different wavelengths in the region 255–260 nm (left). Average values for the change in [θ]MRE were fitted to the Michaelis-Menten equation using GraphPad Prism 6 software to produce the given apparent dissociation constant (right). Stability measurements confirmed that there was no significant change in spectra of the protein alone when continuously measured over a period of 12 h, which was longer than that of an entire titration with spermidine.

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