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Crystallization of *Pseudomonas aeruginosa* AHL synthase LasI using β-turn crystal engineering

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In Gram-negative bacteria, intercellular communication and virulence regulation is mediated by the diffusible chemical signal acyl-homoserine-L-lactone (AHL). The AHL synthase enzymes produce a variety of AHLs from the substrates *S*-adenosyl-L-methionine and acyl-acyl carrier protein. LasI, the AHL synthase from *Pseudomonas aeruginosa*, has low solubility and has failed to crystallize despite extensive crystallization trials. Based on the previously determined structure of the AHL synthase EsaI, active soluble LasI was produced by re-engineering residues in a tight turn to produce a type I′ β-turn. The resulting protein is active, more stable than the wild-type LasI and has been crystallized in the cubic space group $F23$, with unit-cell parameters $a = b = c = 154.90$ Å.

1. Introduction

A wide variety of bacterial phenotypes, including biofilm formation and virulence-factor production, depend on quorum-sensing gene regulation (Costerton *et al.*, 1999; Fuqua *et al.*, 1994). Acyl-homoserine-L-lactones (AHLs) act as bacterial pheromones that allow the bacterium to sense the local cell density and regulate gene expression when a threshold concentration or a 'quorum' of cells is reached (reviewed recently in Miller & Bassler, 2001; Parsek & Greenberg, 2000; Whitehead *et al.*, 2001; Withers *et al.*, 2001) The quorum-sensing system is composed of an AHL signal produced by the AHL synthase and a transcriptional response regulator (reviewed in Fuqua & Greenberg, 2002; Parsek & Greenberg, 2000; Winans, 1988). Inactivating the quorum-sensing regulators may be an effective way to inhibit bacterial quorum-sensing communication and pathogenesis (Rumbaugh *et al.*, 1999).

Crystallographic studies of the *Pseudomonas stewartii* AHL synthase (EsaI) provided the first high-resolution view of the AHL synthases (Watson *et al.*, 2001, 2002). EsaI is from an important plant pathogen that produces the signal 3-oxo-C6-homoserine lactone (Beck von Bodman & Farrand, 1995; Pierson *et al.*, 1999). However, there is also great interest in determining the structure of the AHL synthases of human pathogens such as *P. aeruginosa* (Smith & Iglewski, 2003). LasI is the AHL synthase at the top of the virulence-regulatory hierarchy in *P. aeruginosa* (De Kievit & Iglewski, 2000; Seed *et al.*, 1995). In addition, LasI produces a longer AHL signal, 3-oxo-C12-homoserine lactone (Pearson *et al.*, 1994), and has relatively low sequence identity to EsaI, representing a divergent group of AHL synthases based on sequence analysis.

2. Materials and methods

2.1. Subcloning, mutagenesis, overexpression and mutant protein activity

The gene encoding LasI was subcloned using the *Nde*I and *Bam*HI restriction sites designed in primers used to PCR amplify the gene from genomic DNA into the low-copy T7 expression vector pViet (Hoang *et al.*, 1999), which is an ampicillin-resistance conferring vector that encodes an N-terminal His_6 tag followed by a thrombin-cleavage site adjacent to the inserted gene. LasIΔG was made from pViet-LasI using the QuikChange mutagenesis method (Stratagene) with the following primers: 5′-TTGATCCAGGAAGAT**GGC**CAGGTTTTCGGTTGC-3′ and 5′-GCAACCGAAAACCTG**GCC**ATCTTCCTGGATCAAC-3′. The bases in bold indicate the glycine codon that replaced the TPEA-encoding sequence. The mutation was confirmed using restriction digestion analysis and DNA sequencing of the entire gene.

pViet-LasI and pViet-LasIΔG were transformed into *Escherichia coli* strain SA1503(DE3) and grown in Luria Broth (LB) with 100 µg ml^{-1} ampicillin at 310 K overnight. A 1:50 dilution of the overnight culture was subcultured into 1 l of fresh LB with 100 µg ml^{-1} ampicillin. At a cell density of OD_{600} = 0.6–0.8, the cultures were cooled to 298 K for 15 min and expression was induced by addition of IPTG to a final concentration of 5 m*M*. After ∼8 h, the cells were harvested by

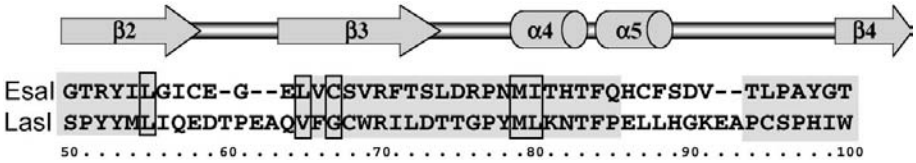

Figure 1
Sequence alignment of LasI with EsaI sequence and structure. The grey blocks denote the conserved sequence blocks of the AHL synthase family and the outlined residues the most conserved amino acids. Cylinders indicate α-helical segments and arrows indicate β-strand regions of EsaI (top sequence) (Watson *et al.*, 2002).

centrifugation at 7000*g* for 10 min and frozen at 253 K for later use.

The His-tagged forms of the LasI and LasIΔG genes were subcloned from the T7 expression vectors into the broad host-range vector pEX30 (Schweizer *et al.*, 2001), where they were now transcribed from the *tac* promoter. After transformation of the resulting plasmids pLasI and pLasIΔG into *E. coli* strain JM109, along with the vector control, AHLs were extracted from the supernatants and quantified using *E. coli* strain MG4/pKDT17 containing the *lasR-lasB-lacZ* detection system (Schaefer *et al.*, 2000). AHL production by *E. coli* harboring vector pEX30, pLasI or pLasIΔG is nearly the same (approximately 90%) as the AHL produced by pLasI expressing wild-type LasI-His_6.

2.2. Purification

Cell pellets from a 3 l overexpression were resuspended in 30 ml lysis buffer (20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl, 5 m*M* imidazole and 1 m*M* β-mercaptoethanol). Treatment with 1 mg ml^{-1} lysozyme on ice for 30 min followed by sonication for 4 $\times$ 45 s ensured sufficient cell lysis. DNaseI was added to a final concentration of 40 µg ml^{-1} for 20 min and the soluble fraction of the lysate was then isolated by centrifugation at 21 000*g* for 25 min at 277 K. This supernatant was incubated and agitated with a 1.5 ml bed volume of washed Ni–NTA resin (Qiagen) pre-equilibrated in lysis buffer at 277 K for 4–6 h. The LasI-bound resin was washed with 5 $\times$ 10 ml of lysis buffer and incubated with 300 units of thrombin in PBS (137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4 pH 7.4) overnight at room temperature with agitation. The soluble protein was collected with 5 $\times$ 1 ml washes of the resin with the lysis buffer. The pooled protein was further purified using Superdex 75 (Amersham Biosciences) size-exclusion chromatography in 20 m*M* Tris pH 7.9, 500 m*M* NaCl and 10 m*M* DTT. The protein was concentrated using a stirred cell (Amicon) to a final concentration of 3.5 mg ml^{-1}. After purification, the LasIΔG protein was >95% pure by Coomassie Blue-stained SDS–PAGE and mass spectrometry (data not shown). The protein was stored by flash-freezing in liquid N_2 and thawed on ice prior to crystallization trials.

2.3. Crystallization and preliminary diffraction

LasIΔG at 3.5 mg ml^{-1} in buffer (20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl and 10 m*M* DTT) was crystallized using a homemade polyethylene glycol-based screen in a hanging-drop vapor-diffusion format. After 3 d at 291 K, small pyramidal crystals appeared with 1.5 *M* $(NH_4)_2SO_4$, 0.1 *M* Li_2SO_4 and 0.1 *M* MOPS pH 6.5, with the addition of 50 µl of 5 *M* NaCl to the well. After optimization, the condition yielding the best diffraction was 1.5 *M* $(NH_4)_2SO_4$, 0.125 *M* Na_2SO_4, 0.1 *M* MOPS pH 6.5 and the addition of 50 µl of 5 *M* NaCl to the well at room temperature with a 6:1 ratio of protein:well solution in the drop. Crystals were transferred to a cryoprotectent [2.0 *M* $(NH_4)_2SO_4$, 0.2 *M* Na_2SO_4, 0.1 *M* MOPS pH 6.5 and 15% glycerol] at 277 K overnight prior to freezing in liquid N_2 for data collection.

Diffraction data were collected on beamline SBC-19ID at the Advanced Photon Source, Argonne National Laboratories and processed using *DENZO* and *SCALEPACK* from the *HKL* suite of programs (Otwinowski & Minor, 1997).

2.4. Stability assays

For the thermal melting CD spectra, LasIΔG was diluted to approximately 15 µ*M* in 20 m*M* Na_2HPO_4/NaH_2PO_4, 250 m*M* NaCl pH 7.8. CD spectra were collected using a Jasco J-810 CD/ORD instrument at a wavelength of 222 nm from 273 to 363 K. For urea and guanidine–HCl denaturation, the proteins were diluted to a final concentration of approximately 25 µ*M* in 0–5 *M* urea or guanidine–HCl. Samples were placed in a 0.5 mm path-length cell and ellipticity was measured at the 222 nm wavelength.

3. Crystal engineering and crystallization

Sequence analysis for the purpose of structure-directed enzyme engineering was conducted after numerous attempts at crystallizing the native LasI protein construct had been unsucessful. Using hidden Markov model sequence analysis as implemented in *HMMER* (Eddy, 1995), multiple sequence alignments of the entire AHL synthase family revealed that LasI contains a four-residue insert in comparison to the 30 other family members. When compared with the sequence and structure of EsaI, this insert was located in a β-turn between strands β2 and β3 (Fig. 1) (Watson *et al.*, 2002). This

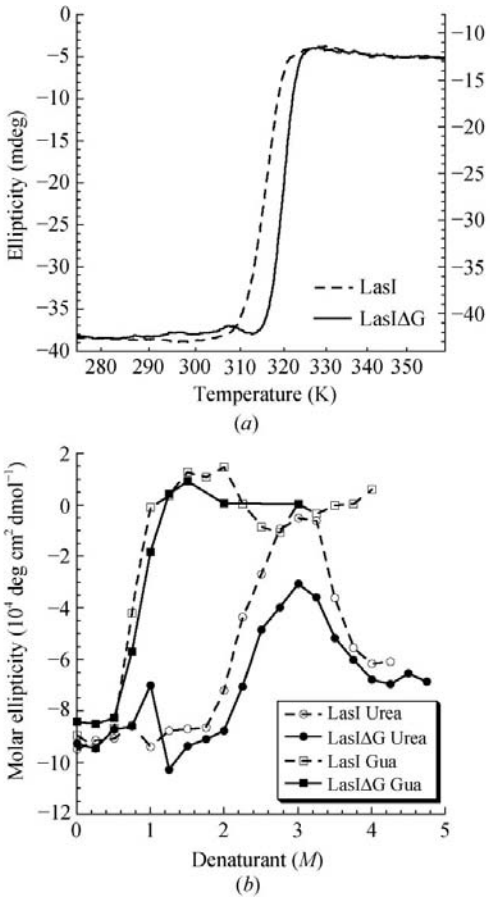

Figure 2
Stability of LasIΔG compared with wild-type LasI enzyme. (*a*) Thermal melting analysis of the native LasI compared with LasIΔG. (*b*) Urea and guanidine denaturation analyses of the native LasI compared with LasIΔG.

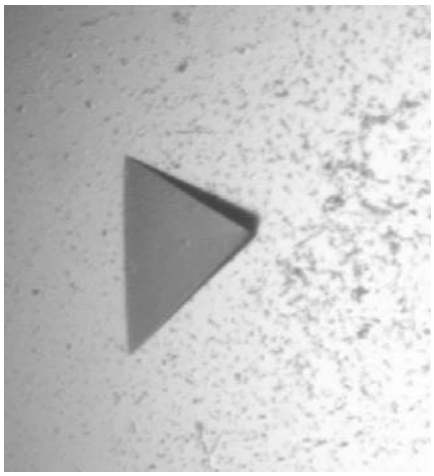

Figure 3
Typical crystal of LasI with dimensions 0.4 × 0.4 × 0.2 mm.

region forms a tight β-hairpin turn in EsaI and is involved in crystal contacts, whereas the loop in LasI would be likely to be larger. Therefore, the region with the four-residue insert, residues 60–63 (TPEA) of the native construct, was replaced with a Gly (residue 60 in LasIΔG) to give a similar loop structure as in EsaI.

The activity and stability of LasIΔG compared with the native LasI were determined. Both the native LasI and LasIΔG produce AHLs when expressed in *E. coli* and the activity of LasIΔG is nearly 100% that of the wild-type enzyme. To determine whether the stability of the mutated protein was altered in comparison to the wild type, a series of protein-stability studies were conducted. Thermal melting analyzed by circular dichroism (Fig. 2*a*) shows that if anything LasIΔG is slightly (4 K) more stable than native LasI. Both proteins denature irreversibly and achieve slightly different denatured states and therefore stability studies were conducted by urea and guanidine denaturation (Fig. 2*b*). With both denaturants, LasIΔG remained folded until higher concentrations of denaturant was used, indicating that it was more stable than the wild-type LasI. However, at the denaturation and melting point both proteins precipitate, forming a β-sheet structure detectable by CD (spectra not shown) (Modler *et al.*, 2003). This β-structure gives an appearance in the CD of some refolding followed by further denaturation at the highest values of temperature and denaturant concentration. All three experiments suggest that the new β-turn sequences inserted into LasI rendered the LasIΔG protein more stable than the wild-type enzyme.

Table 1
Data-collection statistics.

Unit-cell parameter (Å)	154.903
Wavelength (Å)	1.033
Resolution range (Å)	50–2.3 (2.38–2.30)
Completeness (%)	95.0 (76.9)
R_{sym}† (%)	10.3 (22.1)
$\langle I/\sigma(I)\rangle$	25.1 (4.01)
Observed reflections	99109
Unique reflections	13180

† R_{sym} (on intensity) = $\sum\sum|I(h)_i - \langle I(h)\rangle|/\sum\sum I(h)_i$, where $I(h)_i$ is the observed intensity and $\langle I(h)\rangle$ is the mean intensity of reflection h over all measurements of $I(h)$.

Small crystals of LasIΔG were identified by vapor diffusion using a systematic PEG-based crystallization screen. Crystals were optimized (Fig. 3) and diffraction was obtained indicating an *F*23 space group with unit-cell parameters 154.90 Å and one molecule per asymmetric unit, giving a solvent content of 63.8% and a Matthews coefficient of 3.4 Å^3 Da^{-1}. This crystal form is quite different from EsaI (space group $P4_3$; unit-cell parameters $a = b = 66.4$, $c = 47.33$ Å; Watson *et al.*, 2001). The LasIΔG crystals diffract to at least 2.3 Å, as observed during synchrotron data collection. Native data were collected and are of a quality appropriate for structure solution (Table 1).

4. Conclusions

The LasI enzyme, which is important for quorum-sensing regulation of bacterial pathogenesis in the human pathogen *P. aeruginosa*, has been crystallized. A novel crystal-engineering approach was used to obtain the crystals. Swapping of tight turn sequences in LasI to produce LasIΔG proved to be a critical step in producing a more stable enzyme that retains its biological activity and gains the ability to form diffraction-quality crystals.

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