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Crystallization and preliminary crystallographic characterization of the origin-binding domain of the bacteriophage λ O replication initiator

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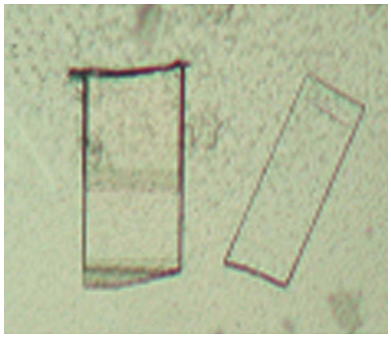


The bacteriophage λ O protein binds to the λ replication origin (*ori*λ) and serves as the primary replication initiator for the viral genome. The binding energy derived from the binding of O to *ori*λ is thought to help drive DNA opening to facilitate initiation of DNA replication. Detailed understanding of this process is severely limited by the lack of high-resolution structures of O protein or of any lambdoid phage-encoded paralogs either with or without DNA. The production of crystals of the origin-binding domain of λ O that diffract to 2.5 Å is reported. Anomalous dispersion methods will be used to solve this structure.

1. Introduction

Bacteriophage λ is the archetype of a family of 'lambdoid' phages. The family members display strong structural homology to one another and share a similar genome organization. λ is a temperate phage that has been intensively studied since its discovery in 1951. These studies have produced groundbreaking insights into important biological processes such as gene regulation, anti-termination of transcription, initiation of DNA replication, site-specific recombination *etc*. (for reviews, see Hendrix *et al.*, 1983; Ptashne, 1992). Bacteriophage λ has served as a valuable model for the elucidation of the biochemical mechanisms involved in the initiation and regulation of DNA replication. Genetic and biochemical studies have demonstrated that the propagation of replication forks along the λ chromosome is carried out entirely by replication proteins encoded by the host (Georgopoulos & Herskowitz, 1971; Kornberg & Baker, 1992; Dodson *et al.*, 1986; Mensa-Wilmot *et al.*, 1989; Zylicz *et al.*, 1989). Bacteriophage λ encodes just two replication proteins: the products of the phage *O* and *P* genes (Joyner *et al.*, 1966; Furth & Wickner, 1983). Both λ replication proteins function in the initiation stage of viral DNA replication (Furth *et al.*, 1978; Dodson *et al.*, 1985; Alfano & McMacken, 1989).

The O protein was found to bind to a series of four repeating DNA sequences (iterons) located within the λ replication origin, *ori*λ (Tsurimoto & Matsubara, 1981; Roberts & McMacken, 1983; Zahn & Blattner, 1985). The capacity of O to bind specifically to *ori*λ is a property of its N-terminal 162 amino acids (Wickner & Zahn, 1986). Following binding of O to *ori*λ and subsequent O self-assembly reactions, the *ori*λ DNA is wrapped around O to form the 'O-some' (Echols *et al.*, 1984). Within the O-some, the origin DNA is sharply bent (∼85° at each iteron; Zahn & Blattner, 1985; Um, 1992), with a total bending approaching 360° for the entire four-iteron site. The binding energy of the assembly of the O-some at *ori*λ induces localized unwinding of a segment of origin DNA (Schnos *et al.*, 1988), which is thought to stimulate initiation of DNA replication by providing an entry point for the replication machinery. A full understanding of how λ O functions in replication initiation must await determination of high-resolution structures of O and of its complex with origin DNA. Unfortunately, certain adverse biophysical properties of O, which include a low solubility at moderate ionic strength and a strong propensity to form higher order aggregates, have made it difficult to obtain high-resolution structures despite numerous attempts over the past two decades. We now report that we have obtained crystals of a truncated form of the N-terminal domain of λ O. We are presently in the process of solving this structure using

anomalous dispersion methods. It has been established that an amino-terminal portion of λ O (residues 1–162) is responsible for the specific binding of O to *ori*λ (Wickner & Zahn, 1986); thus, we will refer to this region of O as the λ origin-binding domain or the λ OBD.

2. Materials and methods

2.1. Expression and purification of the λ OBD

A previous study from this laboratory demonstrated that a protein fragment encompassing λ O amino-acid residues 19–139 contained the specific origin-recognition and dimerization functions of the full-length O protein (Um, 1992). To express this λ OBD more efficiently, a DNA segment that encodes O amino acids 19–139 was amplified by PCR from pRLM4 DNA (Wold *et al.*, 1982), a plasmid template that carries a wild-type λ *O* gene. The amplified DNA was inserted into a multicloning site on a modified pET12a plasmid (Novagen) to produce plasmid pRLM231. DNA-sequence analysis was used to identify pRLM231 clones whose *O* gene sequences were free of mutation. Plasmid pRLM231 DNA was prepared and digested with *Bam*HI and *Xho*I to produce the fragment that encodes the λ OBD 19–139. This fragment was inserted into the expression site of pET21(+) plasmid DNA (Novagen) that had been digested with *Bam*HI and *Xho*I restriction enzymes, yielding plasmid pRLM370. During characterization of the properties of λ OBD expressed from pRLM370, we discovered that the $O_{19–139}$ OBD had a tendency to multimerize in solution (data not shown). Inspection of the amino-acid sequence of the λ OBD revealed that it contained three cysteine residues, two of which were judged not to be involved in specific DNA recognition (Um, 1992). Moreover, these latter two cysteine residues apparently reside within an unstructured region according to the secondary-structure prediction program *PHD* (Rost & Sander, 1993). We thought it possible that formation of nonspecific disulfide linkages might contribute to the tendency of the λ OBD to multimerize. Thus, we modified pRLM370 by site-specific mutagenesis to convert two cysteine codons in *O*, Cys116 and Cys138, to serine codons in individual steps. The resulting plasmid, pRLM373, carries the λ OBD (*i.e.* $O_{19–139}$ C116S, C138S). Plasmid pRLM373 was transformed into *Escherichia coli* BL21(DE3) for production of the 'native' form of the λ OBD and into *E. coli* B834 (a methionine auxotroph; Novagen) for expression of a selenomethionine-modified form of the λ OBD.

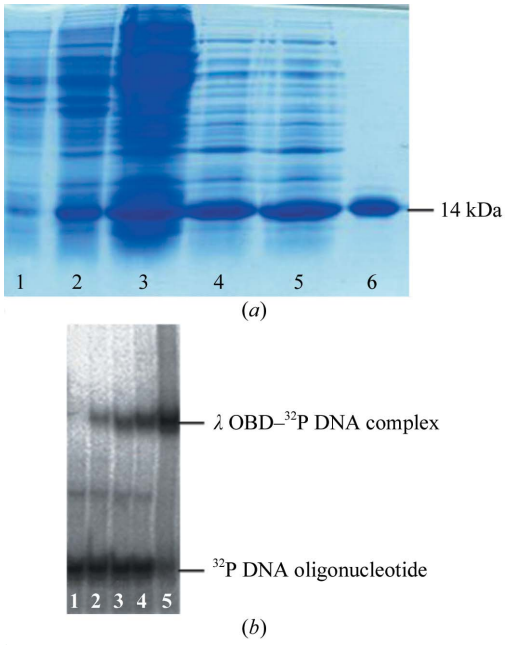


Figure 1
(*a*) Analysis of the purification of the 'native' λ OBD by SDS–PAGE. The protein samples were electrophoresed through a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, uninduced cells; lane 2, induced cells; lane 3, fraction I; lane 4, fraction II; lane 5, fraction II (post-dialysis); lane 6, fraction III. (*b*) Gel mobility-shift assay of binding of the 'native' λ OBD to an oligonucleotide containing a λ O recognition sequence (see text). Increasing amounts of the λ OBD were incubated with a ^{32}P-labeled oligonucleotide at 303 K and the mixture was then subjected to electrophoresis in a native 8% polyacrylamide gel. The molar ratios of protein, as dimer, to DNA were lane 1, 0 (*i.e.* no protein added); lane 2, 0.2; lane 3, 0.5; lane 4, 1; lane 5, 5.

E. coli cells harboring pRLM373 were grown with vigorous aeration at 310 K in rich broth supplemented with 100 µg ml^{-1} ampicillin to express the 'native' form of the λ OBD and in M9 medium supplemented with 100 µg ml^{-1} ampicillin and 50 µg ml^{-1} selenomethionine (Doublié, 1997) to express the Se-containing form of the λ OBD. Expression was induced with 0.5 m*M* IPTG at an optical density of 1.0 at 600 nm and aeration was continued for an additional 3 h (overnight at 310 K for the selenomethionine form). The cell suspension was chilled to 277 K and the cells were harvested by centrifugation. The final cell pellet was resuspended in an equal weight of buffer *R* (40 m*M* HEPES–KOH pH 7.6, 10 m*M* $MgCl_2$) supplemented with protease inhibitors. The cell suspension was lysed using either a lysozyme–heat lysis protocol or sonication. The cell lysate was clarified by centrifugation at 277 K for 60 min at 40 000 rev min^{-1} (120 000*g*) in a Beckman 45 Ti rotor (fraction I, Fig. 1*a*). All purification steps were at 277 K, unless specified otherwise. The clarified cell lysate was mixed with an equal volume of ice-cold ethanol and stirred for at least 3 h. After this step, the 'native' and selenomethionine forms of the λ OBD remain mostly soluble. Insoluble proteins and nucleic acids were removed by centrifugation at 13 000 rev min^{-1} for 20 min in a Sorvall SS34 rotor. The supernatant was collected (fraction II, Fig. 1*a*) and dialyzed overnight against buffer *D* (50 m*M* HEPES, 50 m*M* NaCl, 1 m*M* EDTA) and then applied onto a 30 ml SP-Sepharose column. The bound λ OBD was eluted with a ten-column-volume gradient from 0.05 to 0.5 *M* NaCl. Protein fractions that contained the λ OBD at an estimated purity of ≥98% were pooled (fraction III, Fig. 1*a*) and dialyzed against 100 m*M* NaCl. Finally, both 'native' and selenomethionine-containing preparations were concentrated to ∼10–15 mg ml^{-1}, flash-frozen in liquid nitrogen and stored at 188 K. Typical purification yields were 4–6 mg of protein per litre of culture.

2.2. DNA-binding activity assay

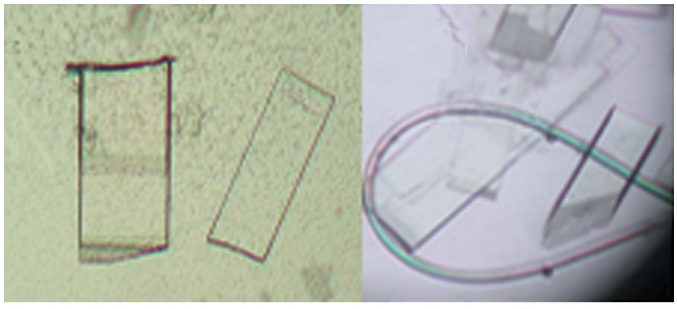
To confirm that the purified λ OBD retained the capacity to bind specifically to *ori*λ, gel-electrophoresis mobility-shift assays were performed under native conditions using an 8–15% polyacrylamide gel in TGE buffer (25 m*M* Tris, 190 m*M* glycine, 1 m*M* EDTA pH 8.3). For this purpose, a 21 bp oligonucleotide with the sequence of iteron III from the λ replication origin (Denniston-Thompson *et al.*, 1977) was chemically synthesized, end-labeled with ^{32}P and tested for binding to the 'native' λ OBD. Binding was observed at roughly equivalent protein and DNA concentrations (Fig. 1*b*).

2.3. Crystallization

Hanging-drop vapor-diffusion experiments were set up using commercial sparse-matrix screens (Hampton Research; Jancarik & Kim, 1991; Cudney *et al.*, 1994) at both room temperature and at 277 K. A reservoir volume of 750–1000 µl, a drop volume of 2–4 µl and a 1:1 macromolecule:well buffer solution ratio were used. Initial

microcrystals (less than 10 µm in any direction) were obtained at 277 K using Crystal Screen condition No. 43 (30% PEG 1500) 5 d after setting up the crystal tray. A solution similar to condition No. 43 was then prepared and used concomitantly with Detergent Screen 1 to optimize the crystallization condition. Very thin crystal plates measuring 30 × 30 µm appeared after 3–5 d at 277 K in various detergents. The optimized crystallization condition contained 25% PEG 1500, 0.1 *M* sodium acetate buffer pH 4.6 and 10% DMSO. Under this condition, crystal plates with a lamellar morphology (evidenced by striations in their thinnest dimension) measuring approximately 70 × 150 × 20 µm formed in 3–5 d (Fig. 2*a*).

Crystals of the selenomethionine-substituted λ OBD were obtained under conditions similar to those for the 'native' form of the protein, but with a slightly modified protocol. The hanging drops were allowed to equilibrate at 277 K for 0.25–24 h. Subsequently, nuclei from the 'native' crystals of the λ OBD were introduced *via* microseeding (*i.e.* streak-seeding; Stura & Wilson, 1990) using a wand consisting of an extensively washed cat whisker attached to a 100 µl pipette tip. Crystals identical in appearance to those yielded by the 'native' λ OBD formed in less than one week (Fig. 2*b*).



(*a*) (*b*)

Figure 2
Crystals of 'native' and selenomethionine forms of the λ OBD. (*a*) Crystals of 'native' λ OBD. (*b*) Mounting a crystal of the selenomethionine-containing λ OBD using a 0.3 mm loop. Crystals measure 70 × 150 µm and are 20 µm thick.

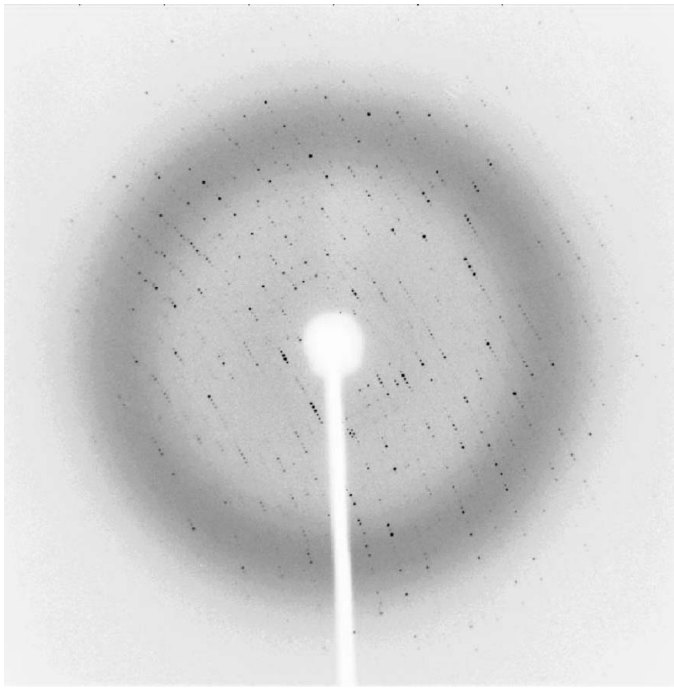


Figure 3
Diffraction image obtained from a crystal of selenomethionine-containing λ OBD. These crystals diffract to a 2.5 Å resolution limit.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	λ_i (inflection)	λ_p (peak)	λ_r (remote)
Wavelength (Å)	0.97953	0.97940	0.96486
Maximum resolution (Å)	2.5	2.5	2.5
Total reflections	56983	64086	39735
Unique reflections	13991	14217	11771
Average redundancy	4.1	4.5	4.1
Completeness (%)	89.4 (52.7)	82.2 (45.1)	68.6 (13.4)
R_{sym}† (%)	7.1 (15.2)	8.5 (16.2)	6.7 (19.7)
$I/\sigma(I)$	16.0 (6.2)	13.5 (6.2)	16.1 (2.2)
Crystal space group	$P2_12_12$		
Unit-cell parameters (Å, °)	$a = 81$, $b = 164$, $c = 35$, $\alpha = \beta = \gamma = 90$		

† $R_{sym} = \sum|I_h - \langle I_h\rangle|/\sum I_h$, where I_h is the measured intensity for an individual reflection and $\langle I_h\rangle$ is the average intensity for that reflection.

Crystals of the λ OBD were flash-cooled in liquid nitrogen prior to X-ray exposure. It is notable that the crystal-growth conditions are intrinsically cryoprotective and were not altered before cryocooling the crystals. Crystals of the 'native' λ OBD diffracted to 2.8 Å at a home-source X-ray generator (data not shown), whereas crystals of the Se-containing λ OBD diffracted to 2.5 Å at a synchrotron source (Fig. 3).

2.4. Data collection

For crystals of the selenomethionine-substituted λ OBD, X-ray diffraction data were collected at the Structural Biology Center, Beamline 19ID, Advanced Photon Source, Argonne National Laboratory, Chicago, IL, USA. The following protocol was used: 0.2° oscillations, 1 s exposure for a total rotation of the crystal of 180° at 120 K for three wavelengths: peak (λ_p, 0.97940 Å), inflection (λ_i, 0.97953 Å) and remote (λ_r, 0.96486 Å). Appropriate wavelengths were determined using selenium X-ray fluorescence (Cromer & Liberman, 1981, and references therein). Diffraction data were collected successively from peak wavelength to inflection to remote wavelengths and were recorded on a CCD detector (ADSC Quantum 315) (Fig. 3). Data were then processed using *HKL*-2000 (Otwinowski *et al.*, 2003) as well as *ImportScaled* and *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994; Potterton *et al.*, 2003). Data-collection statistics are presented in Table 1.

3. Results and discussion

We have obtained crystals of the 'native' and selenomethionine-substituted forms of the λ OBD (*i.e.* λ $O_{19–139}$ C116S, C138S). In addition, we have collected a complete diffraction data set (at Se peak, inflection and remote wavelengths) for the selenomethionine-substituted λ OBD. These crystals diffract to 2.5 Å with good statistics (Table 1). Matthews coefficient and self Patterson map examinations are consistent with the presence of two λ OBD dimers per asymmetric unit.

'Traditional' MAD (Hendrickson & Ogata, 1997) as well as SAD protocols (de La Fortelle & Bricogne, 1997; González, 2003) will be used to attempt to solve the structure.

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Learn (Johns Hopkins Bloomberg School of Public Health) for advice about expression and purification of the λ OBD. Certain commercial materials, instruments and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments or equipment identified are necessarily the best available for the purpose.

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