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# Purification, crystallization and preliminary crystallographic analysis of the biotin–protein ligase from *Pyrococcus horikoshii* OT3

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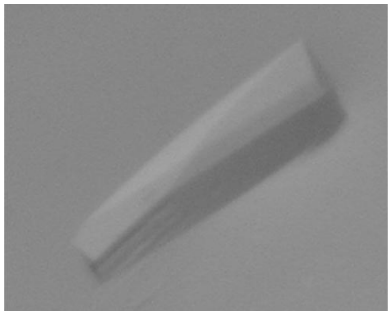



Biotin–protein ligase is an enzyme that catalyzes the ATP-dependent biotinylation of a specific lysine residue in acetyl-CoA carboxylase. The biotin–protein ligase from *Pyrococcus horikoshii* OT3 has been cloned, overexpressed and purified. Crystallization was performed by the microbatch method or the vapour-diffusion method using PEG 2000 as a precipitant at 295 K. X-ray diffraction data have been collected to 1.6 Å resolution from a native crystal and to 1.55 Å resolution from a selenomethionine-derivative crystal for multiple anomalous dispersion phasing using synchrotron radiation at 100 K. The native crystal belongs to the monoclinic space group $P2_1$, with unit-cell parameters $a$ = 38.601, $b$ = 78.264, $c$ = 70.147 Å, $\beta$ = 101.48°. Assuming a homodimer per asymmetric unit gives a $V_M$ value of 2.14 Å$^3$ Da$^{-1}$ and a solvent content of 42.5%. Cocrystals with biotin, ADP and biotinyl-5′-AMP were prepared and diffraction data sets were collected to 1.6, 1.6 and 1.45 Å resolution, respectively.

## 1. Introduction

Biotin–protein ligase (BPL) mediates the biotinylation of acetyl-CoA carboxylase *via* the covalent attachment of biotin to a specific lysine residue of the biotin carboxyl carrier protein (BCCP), which is a core subunit of the carboxylase (Chapman-Smith & Cronan, 1999). This is a two-step reaction: adenylated biotin is first synthesized from the substrates biotin and ATP and the biotin moiety is then transferred to the specific lysine residue of BCCP. The reaction is highly conserved in all organisms, which is reinforced by the fact that BPLs from different species are able to recognize and correctly biotinylate carboxylases from widely divergent sources (Cronan & Wallace, 1995; Tissot *et al.*, 1996). A multienzyme complex, acetyl-CoA carboxylase, catalyzes key reactions in the first committed step in fatty-acid biosynthesis, in which malonyl-CoA is formed from acetyl-CoA, $Mg^{2+}$ATP and bicarbonate (Wakil *et al.*, 1983). In agreement with the biotin-dependent character of these reactions, not only the carboxylase enzymes but also the BPL is essential for survival (Chapman-Smith *et al.*, 2001). The genomes of *Clostridium acetobutylicum*, *Lactococcus lactis*, *Halobacterium* sp., *Pyrococcus abyssii* and *P. furiosus* have two BPL paralogues with and without the N-terminal DNA-binding domain (Rodionov *et al.*, 2002). When BPL contains the N-terminal domain it can also act as a transcriptional repressor that regulates the biosynthesis of biotin. To date, only the crystal structure of BPL from *Escherichia coli* (*Ec*BirA) has been reported (Wilson *et al.*, 1992). *Ec*BirA, a bifunctional enzyme repressor, consists of three domains of 35.3 kDa in total: an N-terminal helix–turn–helix DNA-binding domain, a central catalytic domain and a C-terminal domain (Becket & Matthews, 1997). The catalytic and C-terminal domains of *Ec*BirA show 32% sequence identity to the BPL from *P. horikoshii* OT3 (*Ph*BPL). The amino acids consistent with the N-terminal 60 residues of *Ec*BirA reported as a DNA-binding domain are absent in *Ph*BPL. Therefore, in contrast to *Ec*BirA, *Ph*BPL is considered to be an enzyme with no transcriptional regulatory function. In this paper, we report the expression, purification and preliminary crystallographic analysis of *Ph*BPL and its compexes with the ligands biotin, ADP and biotinyl-5′-AMP. Knowledge of these three-dimensional structures of BPL could help in understanding the reaction mechanisms of BPL and

**Table 1**
Data-collection statistics.

Values in parentheses correspond to the highest resolution shell.

| | | SeMet derivative | | | Liganded forms | | |
|---|---|---|---|---|---|---|---|
| | Native | Remote | Peak | Edge | Biotin | ADP | Biotinyl-5′-AMP |
| Space group | $P2_1$ | $P2_1$ | | | $P2_1$ | $P2_1$ | $P2_1$ |
| Unit-cell parameters | | | | | | | |
| $a$ (Å) | 38.601 | 38.448 | | | 38.420 | 38.204 | 38.348 |
| $b$ (Å) | 78.264 | 82.892 | | | 82.737 | 82.850 | 82.832 |
| $c$ (Å) | 70.147 | 72.509 | | | 72.711 | 72.572 | 72.785 |
| $\beta$ (°) | 101.48 | 102.89 | | | 103.17 | 103.71 | 102.15 |
| Wavelength (Å) | 1.00000 | 0.96500 | 0.97934 | 0.97972 | 1.00000 | 1.00000 | 1.00000 |
| Resolution range (Å) | 40–1.6 (1.66–1.60) | 40–1.55 (1.61–1.55) | | | 40–1.6 (1.66–1.60) | 40–1.6 (1.66–1.60) | 40–1.45 (1.50–1.45) |
| Total No. of observations | 180825 | 231189 | 230011 | 227613 | 212136 | 199287 | 260536 |
| No. of unique reflections | 51798 | 63300 | 63258 | 62617 | 57337 | 56746 | 74265 |
| Completeness (%) | 96.0 (87.9) | 98.8 (99.9) | 98.6 (99.7) | 97.9 (99.9) | 98.7 (96.0) | 98.2 (90.5) | 94.3 (96.3) |
| Mean $I/\sigma(I)$ | 16.0 (3.7) | 11.5 (3.1) | 11.6 (3.1) | 9.9 (3.0) | 16.0 (4.2) | 17.0 (2.3) | 12.0 (3.6) |
| $R_{\rm merge}$† (%) | 6.1 (37.1) | 7.3 (51.8) | 6.4 (47.5) | 7.3 (50.3) | 6.0 (28.5) | 5.5 (30.9) | 5.2 (37.5) |

† $R_{\rm merge} = \sum_{hkl}\sum_j |I_j(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_j \langle I(hkl)\rangle$, where $I_j(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity of measurement $j$ and the mean intensity of the reflection with indices $hkl$, respectively.

acetyl-CoA carboxylase at the molecular level and what it is that structurally distinguishes between the enzyme *Ph*BPL and the bifunctional enzyme repressor *Ec*BirA.

## 2. Experimental

### 2.1. Protein expression and purification

The biotin–protein ligase from *P. horikoshii* OT3 (*Ph*BPL) used in this study has a molecular weight of 26.07 kDa and consists of 235 amino-acid residues. The plasmid encoding *Ph*BPL was digested with *Nde*I and *Bgl*II and the fragment was inserted into the expression vector pET-11a (Novagen) linearized with *Nde*I and *Bam*HI. *E. coli* BL21 Codon Plus (DE3)-RIL cells were transformed with the recombinant plasmid and grown at 310 K in Luria–Bertani medium containing 50 µg ml$^{-1}$ ampicillin for 20 h. The cells were harvested by centrifugation at 4500*g* for 5 min at 277 K, suspended in 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl, 5 m*M* 2-mercaptoethanol and 1 m*M* PMSF and finally disrupted by sonication and heated at 363 K for 11.5 min. The cell debris and denaturated protein were removed by centrifugation at 20 000*g* for 30 min. The supernatant solution was used as the crude extract for purification. The crude extract was desalted with a HiPrep 26/10 desalting column (Amersham Biosciences) and applied onto a Super Q Toyopearl 650M (Tosoh) column equilibrated with 20 m*M* Tris–HCl pH 8.0 (buffer *A*). After elution with a linear gradient of 0–0.3 *M* NaCl, the fraction containing *Ph*BPL was desalted with a HiPrep 26/10 desalting column with 10 m*M* potassium phosphate pH 7.0. The sample was then applied onto a Bio-Scale CHT-20-I column (Bio-Rad) equilibrated with 10 m*M* potassium phosphate pH 7.0 and eluted with a linear gradient of 10–300 m*M* potassium phosphate pH 7.0. The sample was concentrated by ultrafiltration (Vivaspin, 5 kDa cutoff) and loaded onto a HiLoad 16/60 Superdex 200 prep-grade column (Amersham Biosciences) equilibrated with buffer *A* containing 0.2 *M* NaCl. The homogeneity and identity of the purified sample were assessed by SDS–PAGE (Laemmli, 1970) and N-terminal sequence analysis. Finally, the purified *Ph*BPL was concentrated to 9.87 mg ml$^{-1}$ (based on the absorption coefficient of 24 675 cm$^{-1}$ $M^{-1}$ at 280 nm and the molecular weight of 26 071 Da) using ultrafiltration and stored at 203 K. Selenomethionine (SeMet) substituted protein was prepared in an analogous way and its preparations showed virtually identical results to those of the native protein.

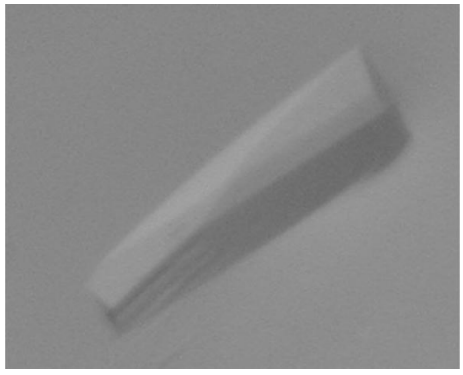

**Figure 1**
Crystals of biotin–protein ligase from *P. horikoshii* OT3. The crystals have approximate dimensions of 0.40 × 0.15 × 0.08 mm.

### 2.2. Dynamic light-scattering study

The oligomerization state of the purified *Ph*BPL was examined by a dynamic light-scattering experiment using a DynaPro MS/X instrument (Protein Solutions). This was performed at a protein concentration of 9.87 mg ml$^{-1}$ in 20 m*M* Tris–HCl pH 8.0 with 500 m*M* NaCl. Several measurements were taken at 291 K and analyzed using the *DYNAMICS* software v.3.30 (Protein Solutions). The result showed a sharp monomodal profile centred at 3.23 nm radius corresponding to a molecular weight of 53.9 kDa, suggesting a dimeric state for *Ph*BPL in solution.

### 2.3. Crystallization and X-ray data collection

Initial crystallization conditions for *Ph*BPL were established using the TERA (automatic crystallization) system (Sugahara & Miyano, 2002) from 144 independent conditions. Crystals were obtained by the microbatch method using Nunc HLA plates (Nalge Nunc International). Each crystallization drop was prepared by mixing 1.0 µl precipitant solution (10.5% PEG 2000, 0.1 *M* acetate–NaOH pH 5.5) and 1.0 µl protein solution at 9.87 mg ml$^{-1}$. The crystallization drop was overlaid with a 1:1 mixture of silicone:paraffin oils, allowing slow evaporation of water in the drop, and stored at 295 K. Higher quality crystals were grown using the sitting-drop method with essentially identical conditions as above. X-ray diffraction data were collected from flash-cooled crystals at 100 K on a Rigaku R-AXIS V image-plate detector using synchrotron radiation at beamline BL26B1 of

SPring-8, Japan. The crystals were flash-frozen using a cryoprotectant solution comprising 10.5% PEG 2000, 0.1 *M* acetate–NaOH pH 5.5 and 20%(*v*/*v*) glycerol. Each frame was exposed for 25 s with a 1° oscillation at a crystal-to-detector distance of 250 mm. Data were processed and scaled using *HKL*2000 (Otwinowski & Minor, 1997).

## 3. Results

We have established the expression, purification and crystallization of PhBPL. Crystals appeared about 4–5 d after setup and grew to approximate dimensions of 0.40 × 0.15 × 0.08 mm after two weeks (Fig. 1). Data-collection statistics are summarized in Table 1. Assuming two chains of *Ph*BPL in the asymmetric unit gives a crystal volume per protein weight ($V_M$) of 2.14 Å$^3$ Da$^{-1}$ and a solvent content of 42.5% (Matthews, 1968). Attempts to obtain a molecular-replacement solution using the crystal structure of *Ec*BirA (Wilson *et al.*, 1992), which shares 32% sequence identity with *Ph*BPL, did not yield any obvious solution. The high-quality selenomethionine-derivative crystals allowed us to apply the multiple anomalous dispersion (MAD) method (Hendrickson *et al.*, 1990) and to obtain the crystal structure of *Ph*BPL at atomic resolution. Crystals also grew in the presence of 5 m*M* concentrations each of biotin, of ADP and of a combination of ATP and biotin, which provided biotin-, ADP- and biotinyl-5′-AMP-bound forms, respectively. The ligand-bound crystals diffracted well and the diffraction data sets were collected (Table 1). To determine the crystal structure, a complete MAD data collection from a SeMet-derivative crystal was performed and the program *SOLVE* (Terwilliger & Berendzen, 1999) was used to find six selenium sites out of the ten possible. On the basis of phases obtained from the MAD experiment, the structure was built and refined and the coordinates have been deposited in the PDB with code 1wq7. The solutions of the liganded forms of the enzyme with biotin, ADP and biotinyl-5′-AMP that correspond to distinct functional states have been obtained by difference Fourier analyses. A detailed discussion of the structures will be published elsewhere.

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