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Cloning, expression, purification and crystallization of dihydroxybutanone phosphate synthase from *Magnaporthe grisea*

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Dihydroxybutanone phosphate synthase (DS) catalyzes a commitment step in riboflavin biosynthesis where ribulose 5-phosphate is converted to dihydroxybutanone phosphate and formate. DS was cloned from the pathogenic fungus *Magnaporthe grisea* (using functional complementation of an *Escherichia coli* DS knockout mutant) and expressed in *E. coli*. The purified protein crystallized in space group $P2_12_12$. Diffraction data extending to 1.5, 1.0 and 1.8 Å resolution were collected from crystals that were divalent cation free, soaked in Zn^{2+} or soaked in Mg^{2+}, respectively.

1. Introduction

In one branch of the riboflavin biosynthetic pathway, 3,4-dihydroxy-2-butanone-4-phosphate synthase (DS) catalyzes the transformation of ribulose 5-phosphate to L-3,4-dihydroxy-2-butanone-4-phosphate (DHBP) and formate (Fig. 1) (Volk & Bacher, 1990, 1991; Richter *et al.*, 1992; Göetze *et al.*, 1998). In the other branch of the biosynthetic pathway, four distinct enzyme-catalyzed steps lead to the generation of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (RAADP) from GTP (Bacher, 1991). The two branches merge at lumazine synthase (LS), which catalyzes the condensation of DHBP with RAADP to yield orthophosphate and 6,7-dimethyl-8-(1′-D-ribityl)-lumazine (DMRL), the immediate precursor of riboflavin (Kis *et al.*, 1995; Jordan *et al.*, 1999; Zheng *et al.*, 2000). Two equivalents of DHBP are incorporated into riboflavin for every one of RAADP. DS and other enzymes of the riboflavin biosynthetic pathway are attractive targets for inhibitor discovery efforts because humans are auxotrophic for riboflavin (vitamin B_2) and obtain this substance through nutrition. In contrast, certain pathogens, particularly Gram-negative bacteria and certain fungi, are unable to obtain sufficient riboflavin from their environment and need to synthesize it in order to survive. The fungus *M. grisea* is the causal agent of rice blast, a perennial disease responsible for enormous losses of grain worldwide. X-ray structures of LS have been solved for three sources, *Bacillus subtilis* (Ritsert *et al.*, 1995), spinach (Persson *et al.*, 1999) and *M. grisea* (Persson *et al.*, 1999), and they have been useful in the design of inhibitors (Cushman *et al.*, 1999*a*,*b*). Although initial efforts towards obtaining an NMR structure of the dimeric (~46 kDa) *E. coli* enzyme have been made (Richter *et al.*, 1999), the three-dimensional structure of DS remains to be elucidated. Here, we report the cloning of DS from *M. grisea*, its expression in *E. coli* and the purification and preliminary crystallographic analysis of the recombinant enzyme.

2. Materials and methods

2.1. Cloning of DS from *M. grisea*

The generation of *E. coli* DS knockout mutants and screening of the *M. grisea* cDNA expression library by functional complementation have been described previously (Bacot *et al.*, 1999). Using this procedure, riboflavin-independent colonies were recovered at a frequency of $\sim 2.1 \times 10^{-5}$. Plasmid DNA was isolated from several representative colonies and the contained cDNA inserts were sequenced completely to yield the predicted full-length amino-acid sequence of *M. grisea* DS (Fig. 2).

2.2. Expression in *E. coli* and enzyme purification

The cloned *M. grisea* DS was modified for insertion into the T7 promoter-based *E. coli* expression vector pET-24a(+) (Novagen),

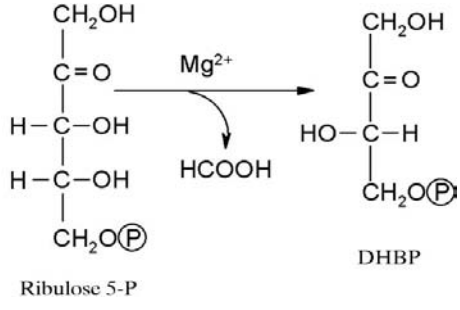

Figure 1
Reaction catalyzed by dihydroxybutanone phosphate synthase.

using primers 5′-CTA CTC ATT TCA TAT GCC TTC CAC AGA CAG CAT-3′ and 5′-CAT CTT ACT AGA TCT TCA ACC CGA CCC ATT CGT C-3′. Following amplification of the target gene, the PCR fragment was cleaved with *Nde*I and *Bgl*II and ligated into the *Nde*I and *Bam*HI sites of the polylinker region of pET-24a(+). The ligation reaction mixture was used to transform a pGroESL-harboring strain of *E. coli* BL21(DE3) using a BTX Transfector 100 (Biotechnologies and Experimental Research Inc.). In the absence of the plasmid pGroESL (Goloubinoff *et al.*, 1989), which confers resistance to chloramphenicol and constitutively overexpresses the *E. coli* GroEL and GroES proteins, *M. grisea* DS is found exclusively in inclusion bodies. A clone was selected for the production of recombinant DS and its plasmid DNA was sequenced completely to check for PCR errors; none were found. The *E. coli* cells were grown in LB media (310 K) containing kanamycin (50 µg ml^{-1}) and chloramphenicol (60 µg ml^{-1}) to an A_{600nm} of ∼1.0, induced for 3 h with 1 m*M* isopropyl-*β*-D-thiogalactopyranoside and harvested.

Purification steps were performed at 273–277 K. The *E. coli* cells containing *M. grisea* DS were suspended in three volumes of 50 m*M* Tris–HCl, 2 m*M* $MgCl_2$ pH 7.5 (buffer *T*), disrupted in a French pressure cell and centrifuged. The supernatant was separated on a column of Q-Sepharose Fast Flow (Pharmacia) equilibrated with buffer *T* and developed with a linear gradient of 0–1 *M* NaCl in buffer *T*. Active fractions were diafiltered extensively against 5 m*M* potassium phosphate, 2.0 m*M* $MgCl_2$ pH 7.5 (buffer *P*) before loading onto a column of hydroxyapatite (Calbiochem) equilibrated with buffer *P*. Active fractions eluted in the column wash with equilibration buffer. Enzyme was concentrated to 7 mg ml^{-1} and diafiltered extensively against 50 m*M* Tris–HCl pH 7.5, frozen in liquid N_2 and stored at 193 K until use. Yield was about 10 mg DS per gram of cell paste. The purified DS was homogeneous as judged by SDS–PAGE and Coomassie blue staining. DS protomer concentrations were estimated using an extinction coefficient at 280 nm of 7270 M^{-1} cm^{-1} as calculated using the GCG *Peptidesort* program (Genetics Computer Group, Madison, WI, USA). Amino-acid sequence comparisons were made using the GCG *GAP* program.

1 MPSTDSIPKS NFDAIPDVIQ AFKNGEFVVV LDDPSRENEA

41 DLIIAAESVT TEQMAFMVRH SSGLICAPLT PERTTALDLP

81 QMVTHNADPR GTAYTVSVDA EHPSTTTGIS AHDRALACRM

121 LAAPDAQPSH FRRPGHVFPL RAVAGGVRAR RGHTEAGVEL

161 CRLAGKRPVA VISEIVDDGQ EVEGRAVRAA PGMLRGDECV

201 AFARRWGLKV CTIEDMIAHV EKTEGKLETN GSG

Figure 2
Amino-acid sequence of dihydroxybutanone phosphate synthase from *M. grisea*.

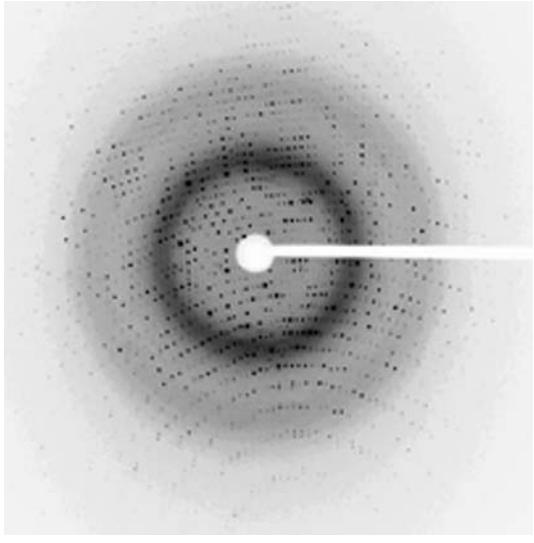

Figure 3
Diffraction pattern of DS crystals soaked in $Zn(OAc)_2$ at beamline 5-ID of the Advanced Photon Source. Conditions: oscillation, 0.5°; exposure, 1.0 s; crystal-to-detector distance, 60 mm; wavelength, 1.0 Å. For presentation, the frame was cropped to produce 1.2 Å resolution at the edge.

2.3. Crystallization

Initial crystals of *M. grisea* DS were obtained at room temperature using the hanging-drop vapor-diffusion technique with screening kits from Hampton Research. *M. grisea* DS (1 µl, 7 mg ml^{-1}) was added to 1 µl of well solution. Optimized well solutions contain 24–30% PEG 5000 monomethyl ether, 0.2 *M* Li_2SO_4, 0.1 *M* MES–NaOH pH 6.0–6.5. Crystals appear within a week, continue growing for two to three months and typically form thin rectangular plates with dimensions 0.2 × 0.2 × 0.05 mm. Divalent cations [200 m*M* $MgCl_2$ or 200 m*M* $Zn(OAc)_2$] were added to the crystals for 8–16 h in soaking solutions in which the PEG 5000 monomethyl ether concentration was increased by 4%. High-quality crystals were also obtained by co-crystallizing the protein with 10 m*M* divalent metal salts in the well solutions described above.

2.4. Data collection and processing

Data sets were collected at the Advanced Photon Source (beamline 5-ID) using a MAR CCD detector or in-house using an R-AXIS IV imaging-plate system with Cu *Kα* X-rays from a Rigaku rotating-anode generator. The crystals were transferred briefly to the well solution enriched with 15% glycerol before flash-freezing in the cold stream (113 or 105 K) generated by an MSC X-stream system used during data collection. Data were processed using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Analysis of the protein

Of the microbial DS homologs known, the *M. grisea* protein (Fig. 2) has the greatest similarity to the *Vibrio harveyi* and *Saccharomyces cerevisiae* proteins at the amino-acid sequence level. The primary structures of DS from *E. coli*, *V. harveyi*, *S. cerevisiae* (GenBank accession numbers X66720, M27139 and Z21619, respectively) and *Ashbya gossypii* (DGene accession number 95N-T03516) are respectively 43.5, 52.7, 52.5 and 48.1% identical to the *M. grisea* DS. Edman degradation of purified recombinant *M. grisea* DS revealed that its first 17 amino acids are identical to those of the protein shown in Fig. 2 except that its initiator Met residue is removed by the bacterial host. Taking this into account, its predicted molecular mass is 24871.88 Da, which is in excellent agreement with the value determined using electrospray ionization mass spectrometry (24870.7 Da). Most importantly, purified recombinant *M. grisea* DS is catalytically active. It exhibited a turnover number of approximately 2.8 min^{-1} (based on enzyme protomer) at 298 K. By way of comparison, the reported turnover number for *E. coli* DS at 310 K is 0.66 min^{-1} (Richter *et al.*, 1992) and that of *Candida guilliermondii* DS at 310 K is 5.6 min^{-1} (Volk & Bacher, 1990).

3.2. Crystallographic analysis

Sulfate ions were present in all conditions examined that produce crystals of *M. grisea* DS, suggesting the possibilities that the

Table 1
Data-collection statistics for crystals of dihydroxybutanone phosphate synthase from *M. grisea*.

Values in parentheses represent the highest resolution range.

Divalent metal salt	$Zn(OAc)_2$	$MgCl_2$	None
λ (Å)	1.00	1.54	1.54
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$
a, *b*, *c* (Å)	53.7, 88.5, 44.0	52.8, 88.0, 43.4	53.7, 87.8, 44.0
Resolution (Å)	1.00 (1.02–1.00)	1.80 (1.83–1.80)	1.50 (1.53–1.50)
No. of observations	686847	72108	110275
Unique reflections	105091	18518	30209
R_{merge} (%)	5.4 (37)	7.3 (26)	4.2 (31)
Completeness (%)	92.4 (60.7)	95.3 (77.0)	88.6 (36)
$I/\sigma(I)$	27.9 (2.0)	19.0 (2.5)	26.7 (3.5)
Packing density ($Å^3\ Da^{-1}$)	2.10	2.03	2.08
Solvent (%)	41	39	41

divalent anions serve either to stabilize the protein or to form contacts in the crystal lattice. Optimized conditions produce crystals of DS in space group $P2_12_12$ that are capable of diffracting to high resolution (Fig. 3). Data sets for divalent cation-free DS crystals and for DS crystals soaked in $MgCl_2$ or $Zn(OAc)_2$ have been collected. Statistics are listed in Table 1. The enzyme requires Mg^{2+} for catalytic activity and it will be of interest to find its location within the active site. Although Zn^{2+} does not support catalytic activity, the 1.0 Å resolution of the data set for the crystal containing Zn^{2+} should allow an unambiguous assignment of the divalent metal binding site. The presence of Zn^{2+} may also allow determination of the phases using multiwavelength anomalous diffraction (MAD) methods (Hendrickson *et al.*, 1990). Assuming one monomer per asymmetric unit results in reasonable values for the packing densities. The protein exists as a dimer in solution. The high-resolution data collected on DS should yield a high-quality three-dimensional structure of the enzyme that will be useful in understanding its catalytic mechanism and designing novel inhibitors that target pathogens.

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