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Cloning, expression, purification and preliminary crystallographic characterization of a shikimate dehydrogenase from *Corynebacterium glutamicum*

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The shikimate dehydrogenase from *Corynebacterium glutamicum* has been cloned into an *Escherichia coli* expression vector, overexpressed and purified. Native crystals were obtained by the vapour-diffusion technique using 2-methyl-2,4-pentanediol as a precipitant. The crystals belong to the centred monoclinic space group *C*2, with unit-cell parameters a = 118.77, b = 63.17, c = 35.67 Å, β = 92.26° (at 100 K), and diffract to 1.64 Å on a synchrotron X-ray source. The asymmetric unit is likely to contain one molecule, corresponding to a packing density of 2.08 Å^3 Da^{-1} and a solvent content of about 41%.

1. Introduction

The shikimate pathway is responsible for the synthesis for aromatic compounds such as the amino acids phenylalanine, tyrosine and tryptophan as well as several other nonaromatic compounds. It is conserved in bacteria (including archea), protists, plants and fungi, but has never been found in metazoic animals (Herrmann & Weaver, 1999). Therefore, enzymes of this pathway are potential targets for antimicrobial (Davies *et al.*, 1994) and antiparasite drugs (Roberts *et al.*, 1998), herbicides (Kishore & Shah, 1988) and fungicides.

Shikimate dehydrogenase (SDH) catalyzes the NADPH-dependent reduction of 3-dehydroshikimate to shikimate, the fourth step in the seven-step shikimate pathway. The 'classical' SDH is named AroE (EC 1.1.1.25). It is highly specific for shikimate and for the cosubstrate NADP. A shikimate dehydrogenase-like enzyme (SDH-L; EC 1.1.1.25) from *Haemophilus influenzae* has been described that has comparable substrate and cosubstrate specificities to those of AroE, but that has a catalytic efficiency that is several orders of magnitude lower (Singh *et al.*, 2005). A presumably bifunctional SDH has also been found in *Escherichia coli* (YdiB; EC 1.1.1.282). This enzyme also reduces quinate to 3-dehydroquinate with either NADP or NAD as a cosubstrate (Benach *et al.*, 2003). NAD is mostly found in catabolic reactions. Quinate is an abundant plant product and can be degraded by many bacteria and fungi (Grund & Kutzner, 1998). The structures of these different SDH subclasses are highly conserved (Singh *et al.*, 2005), even if their sequence identitities are often only between approximately 25 and 35%.

In bacteria, SDH exists as a monomer or a homodimer (Singh *et al.*, 2005; Chaudhuri & Coggins, 1985), while in plants SDH is associated with dehydroquinase as a bifunctional enzyme (Deka *et al.*, 1994) and in fungi it is part of the pentafunctional multienzyme complex catalyzing steps two to six of the shikimate pathway (Lumbsden & Coggins, 1977).

Structural analysis of SDH will provide information about (co)substrate recognition. Not only could it help in the design of new inhibitors, but it could also help in understanding the noticeable differences in the enzymatic properties of the SDH subclasses. Moreover, there is considerable interest in understanding the details of amino-acid metabolism in *Corynebacterium glutamicum* as this

organism is widely used for the synthesis of aromatic compounds in industrial processses.

2. Cloning of the shikimate dehydrogenase

The bacterial chromosome of *C. glutamicum* strain ATCC 13032 was isolated *via* NaOH/SDS cell lysis and P/C/I extraction (Sambrook & Russell, 2001). For polymerase chain reaction (PCR), mutation-introducing oligonucleotide primers (CglSDH-5′-BspHI, 5′-GTA-GAAAGCCCAAAA**TC**ATGAACGACAGTATTCTCCTCGG, and CglSDH-3′-BamHI, 5′-GAAGCGTCGAGGTTTTAC**G**GA**TC**CTT-TTAGAGGGACAGG) were designed based on the complete genome sequence of *C. glutamicum*. Mismatched nucleotides are shown in bold. The PCR products were purified and cleaved with the restriction endonucleases *Bsp*HI and *Bam*HI and inserted into the overexpression vector pNHis (Chatterjee *et al.*, 2005), which had previously been incubated with the enzymes *Nco*I and *Bam*HI. The resulting plasmid pNHIS-SDH was transformed into competent *E. coli* XL1-Blue cells (Inoue *et al.*, 1990) and selected on Luria–Bertani (LB) agar plates containing 150 µg ml^{-1} ampicillin. Single colonies were used to inoculate 4 ml LB medium containing 150 µg ml^{-1} ampicillin. The cells were grown overnight and the plasmid was isolated with a plasmid-preparation mini kit.

Finally, the resulting vector was DNA sequenced by the dideoxy chain-termination method using the oligonucleotide primers T7-promoter (5′-TAATACGACTCACTATAGG) and T7-terminator (5′-GCTAGTTATTGCTCAGCG).

3. Protein expression and purification

Competent *E. coli* strain BL21(DE3) cells were transformed with the pNHIS-SDH vector (Inoue *et al.*, 1990). A volume of 1 l bacterial LB culture containing 150 mg ampicillin was grown to an optical density at 600 nm (OD_{600}) of ∼0.6 at 310 K at 220 rev min^{-1} on an orbital shaker. Overexpression of the enzyme was induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 1 m*M*. The cells were harvested at OD_{600} = 1.2, pelleted by centrifugation and frozen at 193 K. The bacteria were resuspended in 20 ml lysis buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10 m*M* imidazole pH 8.0) and lysed by passage through a French press at 96 MPa three times. The crude lysate was centrifugated at 10 000*g* for not less than 20 min at 277 K to obtain a clarified lysate. For immobilized metal-affinity chromatography, a gravity-flow column containing 2.5 ml Ni–NTA matrix (Qiagen) was equilibrated with 10 ml lysis buffer, charged with the clarified lysate and rinsed twice with 10 ml wash buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 20 m*M* imidazole pH 8.0).

Figure 1
A large crystal (∼1.1 × 0.1 × 0.1 mm) with a smaller one beside it. A sliver from the large crystal was taken for data collection.

Table 1
Crystallographic data statistics.

Resolution shell (Å)	$I/\sigma(I)$	Completeness (%)	R_{sym} (%)
99.00–3.53	34.1	98.9	6.1
3.53–2.80	27.7	98.8	6.9
2.80–2.45	19.5	98.6	9.6
2.45–2.22	15.4	98.5	11.7
2.22–2.06	11.8	98.4	14.4
2.06–1.94	8.0	98.0	18.0
1.94–1.84	5.5	97.7	24.3
1.84–1.76	3.8	97.9	29.7
1.76–1.70	2.8	97.4	37.3
1.70–1.64	1.7	74.5	59.1
All	17.5	95.9	9.5

Afterwards, elution was performed with 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 250 m*M* imidazole pH 8.0. The resulting fractions were analyzed *via* SDS–PAGE using Nu-PAGE 4–12% bis-tris gels. Electrophoresis was performed in SDS running buffer [0.1 *M* MES, 0.1 *M* Tris, 0.2%(*w*/*v*) SDS and 1.6 m*M* EDTA, supplied with antioxidant as described by the supplier Invitrogen]. The protein bands were stained by using Coomassie brilliant blue G250 (Merck). Size-exclusion chromatography of the SDH-containing fraction with a Superdex 200 prep-grade column (Amersham Biosciences) was then performed. The protein samples were concentrated and buffer-exchanged [50 m*M* Tris, 500 m*M* NaCl, 20%(*v*/*v*) glycerol pH 7.5] by ultra-filtration with Amicon Ultra-15 centrifugal filter devices with 10 kDa molecular-weight cutoff (Millipore).

The molecular weight of the expressed protein was analyzed by electrospray ionization–mass spectrometry (ESI–MS) in a Finnigan LCQ MS ion-trap LC-MS. For this purpose, the samples were buffer exchanged to 10 m*M* sodium hydrogen phosphate pH 7.0 by ultra-filtration with Amicon Ultra-15 centrifugal filter devices with 10 kDa molecular-weight cutoff (Millipore) and the protein concentration diluted to 10 µ*M* with the same buffer.

4. Crystallization

Crystals were grown using sitting-drop vapour diffusion at 293 K over 300 µl reservoir containing 100 m*M* sodium actetate buffer pH 4.6, 200 m*M* NaCl, 20%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD). Each drop contained 2 µl NADH (2 µg ml^{-1}), 2 µl reservoir solution and 2 µl protein solution composed of 50 m*M* Tris–HCl, 500 m*M* NaCl, 1 m*M* NaN_3, 20%(*v*/*v*) glycerol pH 7.5 and 3.75 mg ml^{-1} protein as determined by the Bradford method using BSA as a standard (Bradford, 1976). After about five months, a large rod-shaped crystal grew to dimensions of about 1.1 × 0.1 × 0.1 mm amongst some smaller crystals (Fig. 1). The large crystal was fragmented with a needle.

5. X-ray analysis

A high-resolution data set from the crystal was measured at 100 K (Fig. 2) using synchrotron radiation on the wiggler beamline BW7B (λ = 0.843 Å) of the DORIS storage ring at the EMBL Outstation at DESY (Hamburg, Germany). The data set was collected over 180° with 1.2° oscillation per frame on a MAR Research 345 image-plate detector. The distance between the crystal and the detector was 300 mm.

Because of the high intensity of the X-ray beam of the synchrotron, cryocooling is essential. There were sufficient amounts of MPD in the precipitation agent and glycerol in the protein solution that additional soaking of cryoprotectant was not necessary for cryo-data

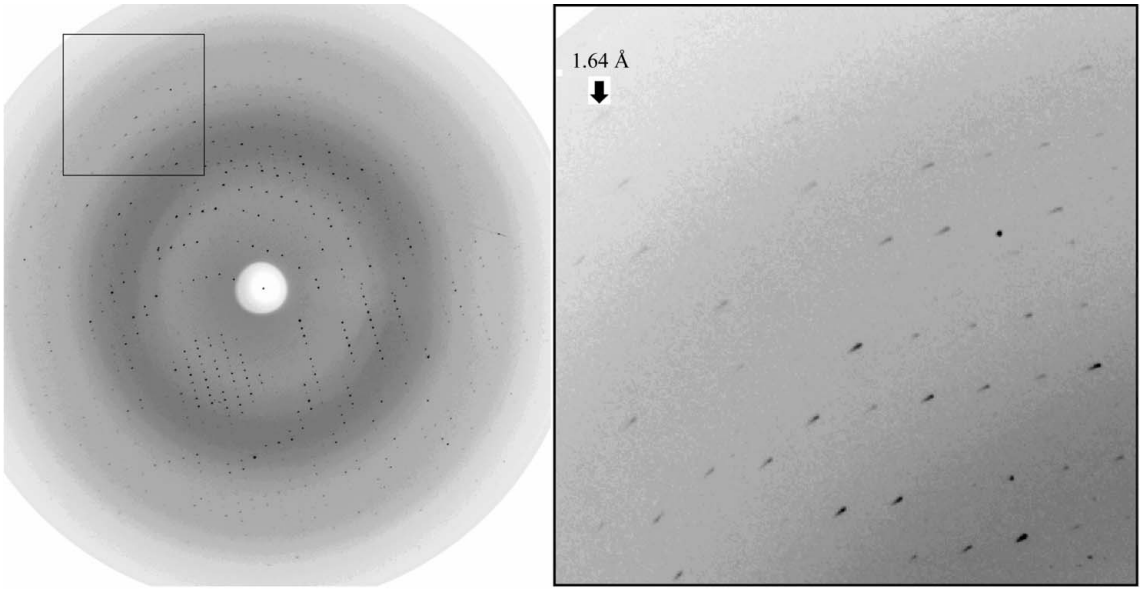

Figure 2
A typical diffraction pattern of SDH collected on beamline BW7B at the DESY in Hamburg. Some spots show a 'tail' caused by the mosaicity of 0.9°.

collection. The crystals were loop-mounted directly from the drop and cryocooled to 100 K using an Oxford Cryosystems Cryostream. Slivers of the large crystal showed the broadest diffraction patterns. A native data set was collected to 1.64 Å. Data were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). From autoindexing with *DENZO*, the crystals were found to index in the centred monoclinic space group *C*2, with unit-cell parameters $a = 118.77$, $b = 63.17$, $c = 35.67$ Å, $\beta = 92.26^\circ$ at 100 K. In space group *C*2, one monomer per asymmetric unit leads to an acceptable packing density V_M (Matthews, 1968) of 2.08 Å^3 Da^{-1}, which corresponds to a solvent content of about 41%. Based on Wilson plot calculations using the program *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994), the *B* factor is 17.25 Å^2. Furthermore, according to the cumulative intensity distributions, no indication of twinning was observed. To obtain the data set, 262 272 reflections were observed, with 32 327 reflections being unique, corresponding to a redundancy of about 8.1. Additional statistics of the native data set are given in Table 1. A full description of the structure determination will be published elsewhere.

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