Escherichia coli dihydrodipicolinate synthase

Identification of the active site and crystallization

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Escherichia coli dihydrodipicolinate synthase (DHDPS) (EC 4.2.1.52), the first enzyme unique to lysine biosynthesis, catalyses the condensation of pyruvate and aspartate β -semialdehyde (ASA) by a ping-pong mechanism. Pyruvate binds first to the enzyme, forming a Schiff base with the ϵ -amino group of Lys-161, followed by binding of ASA. K_m values of 0.57 and 0.55 mM were determined for pyruvate and DL-ASA respectively. 3-Bromopyruvate inhibits DHDPS with a K_i of 1.6 mM. DHDPS is 50% inhibited by 1.0 mM-L-lysine, 1.2 mM-sodium dipicolinate or 4.6 mM-S-2-aminoethyl-L-cysteine. Crystals of DHDPS diffracting to beyond a resolution of 0.24 nm (2.4 Å) were obtained under several experimental conditions. Diffraction patterns were compatible with trigonal space groups $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 12.26$ nm and $c = 11.19$ nm. The density of the crystals indicates the presence of a dimer of DHDPS subunits per asymmetric unit.

INTRODUCTION

Lysine biosynthesis via the diaminopimelate pathway of prokaryotes, some *Phycomycetes* and higher plants starts from aspartate and shares the first two reactions with the synthesis of methionine, threonine and isoleucine [1]. The first specific step of lysine biosynthesis, the condensation of aspartate β -semialdehyde (ASA) and pyruvate to dehydrodipicolinate, is catalysed by dihydrodipicolinate synthase (DHDPS) (Fig. 1).

The complete amino-acid sequences of DHDPS from *Escherichia coli* [2], *Corynebacterium glutamicum* [3], wheat [4] and maize [5] have been communicated and it has been shown that these enzymes exist as homotetramers.

DHDPS isolated from higher plants catalyses the condensation reaction by means of a ping-pong mechanism in which pyruvate forms a Schiff base with a lysine residue in the active site of the enzyme [6,7]. All higher-plant enzymes are strongly feedback-inhibited by low concentrations of lysine in an allosteric manner [6–9], indicating that lysine regulates the metabolite flux through its biosynthetic pathway. Additional evidence for DHDPS as the main regulatory point in higher-plant lysine biosynthesis is provided by the fact that a tobacco mutant with desensitized DHDPS overproduces lysine [10], while all plant mutants with aspartate kinases desensitized to lysine inhibition overproduce threonine [11].

The *E. coli* DHDPS is also inhibited by lysine [12], but to a much lesser extent. However, the physiological role of this feedback inhibition is questionable, since mutants with desensitized aspartate kinase III excrete lysine but not threonine, an unexpected property for cells with efficient regulation of the branch-point enzyme [13]. Anyway, DHDPS must catalyse the rate-limiting step in *E. coli* lysine biosynthesis after aspartate kinase III, because increasing the *dapA* gene (encoding DHDPS) copy number in a *lysC* mutant with desensitized aspartate kinase III results in a parallel increase of DHDPS activity and lysine excretion [14].

In addition to its central role in lysine biosynthesis in higher plants and bacteria, DHDPS also delivers the direct precursor of

dipicolinic acid, which plays a key role in the bacterial sporulation process [15]. Consequently DHDPS has attracted interest as a suitable target for new herbicidal or antimicrobial compounds. Therefore, the full understanding of the reaction mechanism of this enzyme and of its kinetic and regulatory properties is highly desirable and best met by the availability of its three-dimensional structure as provided by X-ray crystallography.

In the present paper we report an improved purification procedure for DHDPS from an overproducing *E. coli* strain, the identification of the active site, and also describe the crystallization and preliminary crystallographic data of the enzyme.

MATERIALS AND METHODS

Materials

E. coli RDA8/pDA3, carrying the *dapA* gene on a multicopy plasmid, was a gift from Dr. C. Richaud (Institut Pasteur, Paris, France). DL-ASA was synthesized by ozonolysis of DL-allylglycine and purified by ion-exchange chromatography [16]. *N*-Tosyl-L-phenylalanylchloromethane ('TPCK')-treated trypsin was purchased from Sigma.

Enzyme assay

The assay mixture contained 100 mM-imidazole buffer, pH 7.4, 10 mM- K_2HPO_4 , 5 mM-pyruvate, 2 mM-DL-ASA and enzyme in a volume of 1 ml. The reaction was started by the addition of enzyme and the increase in absorbance at 270 nm was measured at 37 °C against a blank without enzyme [16a], giving a linear

Fig. 1. Reaction catalysed by DHDPS

Abbreviations used: DHDPS, dihydrodipicolinate synthase(s); ASA, aspartate β -semialdehyde; OG, *N*-octyl- β -D-glucopyranoside.

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reaction rate after a short lag phase. One unit of DHDPS gave an increase in absorbance of 1.0/min (corresponding to about 5 nmol of tetrahydrodipicolinate formed/s).

Enzyme purification

Crude extract. Bacteria cultured in 5 l of Luria–Bertani medium were harvested by centrifugation (approx. 30 g wet wt) at 10000 *g* for 10 min, resuspended in 20 mM-Tris, 10 mM-potassium pyruvate, 100 mM-KCl, pH 8.0, (approx. 3 ml/g wet wt) and disrupted by sonification (25 × 30 s). After centrifugation (15 min, 15000 *g*), the supernatant was collected and the sonification procedure repeated twice.

Heat treatment. The combined supernatants were transferred to test tubes (approx. 14 mm i.d.) which were immersed for 2 min in a water bath at 70 °C and then immediately cooled on ice. Precipitated proteins were removed by centrifugation (15 min, 15000 *g*).

DEAE-Sepharose chromatography. The supernatant was applied to a DEAE-Sepharose column (5 cm × 13 cm) which had been equilibrated with 20 mM-Tris/100 mM-KCl, pH 8.0. After extensive washing the column was eluted with a linear gradient of 0.1–0.5 M-KCl in 20 mM-Tris, pH 8.0.

Phenyl-Sepharose chromatography. Active fractions were pooled, adjusted to a concentration of 0.5 M-ammonium sulphate by the addition of solid ammonium sulphate and applied to a phenyl-Sepharose column (2.4 cm × 17 cm, equilibrated with 0.5 M-ammonium sulphate in 20 mM-Tris, pH 8.0). After washing with equilibration buffer, the enzyme was eluted with 20 mM-Tris, pH 6.0. Fractions containing enzyme activity were pooled and dialysed against 20 mM-Tris, pH 8.0.

F.p.l.c. on Q-Sepharose. F.p.l.c. was done with Pharmacia equipment and a Pharmacia Q-Sepharose fast-flow column. About 50 mg of protein (a third of the total sample) was applied to a Q-Sepharose column (HR 16/10, 1.6 cm × 10 cm) equilibrated with 20 mM-Tris, pH 8.0, and was eluted with 235 ml of a linear gradient from 0 to 0.5 M-KCl in 20 mM-Tris, pH 8.0. DHDPS purified in three successive runs was pooled, concentrated by ultrafiltration (Amicon PM 10 membranes) and, after the addition of 0.57 vol. of glycerol, stored at –20 °C for several months without loss of activity. A summary of the purification procedure is presented in Table 1.

Molecular mass estimations

The molecular mass of the enzyme was estimated by SDS/PAGE [17] and by gel filtration using a Pharmacia Ultropac TSK-G3000-SW f.p.l.c. column equilibrated with 20 mM-Tris/100 mM-KCl, pH 8.0. Carbonic anhydrase and monomers and dimers of BSA and ovalbumin were used to calibrate the column.

Protein concentration determination

Protein concentrations were determined by the method of Bradford [18] using BSA as the standard. The concentration of homogeneous enzyme was calculated based on an absorption coefficient at 280 nm of 0.412 l·mg^{–1}·cm^{–1}.

Peptide mapping

Either 150 µl of 100 mM-pyruvate or 150 µl of water were added to 1 ml samples of purified DHDPS (18 mg/ml in 20 mM-Tris, pH 8.0). After 10 min at room temperature, 30 µmol of NaBH₄ was added to both samples. After 4 h on ice the reaction mixtures were desalted on Pharmacia PD-10 columns (equilibrated with 50 mM-NH₄HCO₃) and freeze-dried. Freeze-dried DHDPS was denatured, mercaptolysed and then alkylated with 4-vinylpyridine [19]. After digestion with trypsin [20]

Table 1. Purification of DHDPS from *E. coli*

Purification step	Volume (ml)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	163	4091	11	100	1
Heat treatment	140	545	71	89	6.7
DEAE-Sepharose	350	246	142	81	13.3
Phenyl-Sepharose	61	148	162	54	15.1
Q-Sepharose	41	100	270	62	25.2

fragments were separated on a Macherey–Nagel ET 250/8/4 Nucleosil 300-10 C-18 h.p.l.c. column in an elution gradient from 0 (solvent A) to 80% acetonitrile (solvent B) in 0.1% trifluoroacetic acid. Peptides were further separated by rechromatography on the C-18 h.p.l.c. column in an elution gradient from 0 (solvent A) to 50% acetonitrile (solvent B) in 50 mM-potassium phosphate buffer, pH 6.25.

Amino-acid and sequence analysis

Amino-acid analyses were carried out on a Biotronic LC 600 amino-acid analyser after hydrolysis with 6 M-HCl at 110 °C for 24 h. Peptides were sequenced by Edman degradation in a prototype spinning-cup sequenator [21,22].

Crystallization

Purified DHDPS was passed through a Pharmacia PD-10 gel-filtration column, the buffer being exchanged against 2 mM-Mes, pH 6.8. Crystallization experiments were performed with and without effectors at room temperature and at 4 °C using sitting-drop vapour diffusion. Ammonium sulphate (1.25–3.0 M), sodium/potassium phosphate (1.25–3.0 M), sodium citrate (0.8–1.6 M) and phosphate (0.2–1.5 M)/10% (w/v) PEG 6000 as precipitating agents, were assayed in the pH range from 4 to 10.

It has been shown [23] that DHDPS loses its enzymic activity irreversibly when treated with NaBH₄ in the presence of sodium pyruvate. For crystallization experiments the enzyme was inactivated as follows: 990 µl of DHDPS solution (activity 160.8 units/ml) was incubated with 25 µl of 0.1 M-sodium pyruvate in 0.1 M sodium/potassium phosphate buffer, pH 7.0, for 10 min at 20 °C. Aliquots (25 µl) of 0.2 M-NaBH₄ solution in the same buffer were added under cooling in an ice bath. After 2 h, the reaction mixture was chromatographed using a Pharmacia NAP5 column. The assay for enzymic activity yielded a value of 1.3 ± 0.5% residual activity.

Crystal density determination

The density of the crystals was determined by the Bode–Schirmer Ficoll density-gradient method [24].

Data collection

X-ray intensity data from trigonal crystals grown at basic pH in the presence or absence of inhibitors and of a mercury methyl chloride derivative were collected using a FAST television area diffractometer (Enraf–Nonius, Delft, Holland) with CuK_α X-radiation from a Rigaku Denki generator operated at 5.4 kW. A 0.24 nm (2.4 Å) native data set was collected and processed using MADNES software [25], applying specifications described in [26]. Additionally, diffraction results were collected on film with a rotation-precession camera (Huber, Rimsting, Germany) at –4 °C (because of radiation sensitivity of the crystals). Film data were evaluated with the FILME program ([27] modified by S. Bennett).

Fig. 2. Reversed-phase h.p.l.c. separation of tryptic peptides of DHDPS (a) and of DHDPS reduced with NaBH_4 in the presence of pyruvate (b)

RESULTS AND DISCUSSION

Purification and molecular mass of the enzyme

In a typical purification procedure, summarized in Table 1, DHDPS was purified 25-fold to a specific activity of 270 units/mg of protein with 62% yield. Starting from 30 g of *E. coli* wet cell paste approx. 100 mg of purified DHDPS was obtained. Addition of 10 mM-pyruvate to the crude extract greatly increased the recovery of enzyme activity during the heat treatment.

The purified DHDPS (native or pyruvate/ NaBH_4 -inactivated) showed a single band upon SDS/PAGE corresponding to a molecular mass of 32 ± 2 kDa. Since the molecular mass of the native enzyme was determined to be 112 kDa by gel filtration, DHDPS is probably a tetramer composed of four subunits with identical molecular masses.

An isoelectric point of approx. 4.5 was determined by isoelectric focusing for both native and inactivated enzyme.

Identification of the active site

Since the DHDPS reaction is known to proceed via Schiff base formation between the carbonyl group of pyruvate and an ϵ -amino group of a lysine residue [23], experiments were carried out to identify this lysine residue in the polypeptide chain. Treatment of DHDPS for 4 h with NaBH_4 in the presence of pyruvate resulted in a 98.7% loss of enzyme activity (pyruvate-inactivated enzyme), while DHDPS treated with NaBH_4 in the absence of pyruvate (control) had lost only 1.8% of its activity.

Control and pyruvate-inactivated DHDPS were denatured, reduced with mercaptoethanol and alkylated with 4-vinylpyridine. After digestion with trypsin the resulting peptide (P-1–P-27) were separated by reversed-phase h.p.l.c. and identified by amino-acid analysis. Comparison of peptide maps obtained from control and pyruvate-inactivated DHDPS (Fig. 2) revealed three major differences. In the map of pyruvate-inactivated enzyme (Fig. 2b) peaks corresponding to P-14 (Glu-162–Arg-169) and P-13 (Asn-156–Lys-161) were missing, while the peak containing P-23 (Glu-258–Arg-268) in the control (Fig. 2a) had increased in size after pyruvate inactivation (P-X).

The amino-acid composition of P-X was in agreement with the

Table 2. Amino acid compositions of tryptic peptides P-13, P-14, P-23 and P-X in comparison with those deduced from the corresponding DHDPS sequence [2]

Values in parentheses are deduced from the corresponding DHDPS sequence.

Amino acid	P-13	P-14	P-23	P-X
Asp/Asn	1.1 (1)	1.0 (1)	1.0 (1)	2.8
Thr	–	1.9 (2)	1.9 (2)	3.7
Glu/Gln	–	1.1 (1)	1.1 (1)	2.1
Gly	1.0 (1)	0.9 (1)	0.9 (1)	2.5
Ala	–	1.1 (1)	1.1 (1)	2.0
Val	–	–	1.0 (1)	1.1
Ile	1.7 (3)	–	–	1.5
Leu	–	1.0 (1)	2.9 (3)	4.0
Lys	0.9 (1)	–	–	–
Arg	–	1.0 (1)	1.0 (1)	2.1

Fig. 3. Rechromatography at neutral pH on a reversed-phase h.p.l.c. column of tryptic peptides P-23 of DHDPS (a) and P-X of DHDPS reduced with NaBH_4 in the presence of pyruvate (b)

sum of P-13, P-14 and P-23 with the exception of the single lysine (Lys-161) of P-13. This residue could not be identified; instead a peak of ninhydrin-positive material eluted from the analyser with a retention time different from any of the 20 protein amino acids (Table 2).

Upon rechromatography with a mobile phase of nearly neutral pH (Fig. 3), material from P-23 eluted as a single peak (Fig. 3a), but material from P-X produced two fragments (P-X1 and P-X2) (Fig. 3b). Amino-acid analysis revealed that the composition of P-X1 was in agreement with the composition expected for P-23. The composition of P-X2 agreed with the composition expected

the precipitating agent and 6% (w/v) *n*-octyl- β -D-glucopyranoside (OG), equilibrated against 1.8–2.0 M-sodium/potassium phosphate, well-formed trigonal crystals grew up to 0.8 mm in their largest dimension and up to 0.6 mm wide in about one week. These crystals were suitable for X-ray diffraction analysis, diffracting to beyond 0.24 nm (2.4 Å) resolution. Diffraction patterns were compatible with trigonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 12.26$ nm and $c = 11.19$ nm. The density of the crystals was determined as 1.105 ± 0.004 g/cm³, which corresponds to a dimer of DHDPS subunits in the asymmetric unit. The tetrameric molecule is located on the twofold axis. The volume per unit-protein molecular mass, V_M , is 0.381 nm³/Da (3.81 Å³/Da). The corresponding solvent content of 68% is slightly higher than commonly observed in other protein crystals (27–65%) [30]. Trigonal crystals of the same space group and with similar cell constants were obtained when the crystallization experiments were performed in the presence of inhibitors (bromopyruvate, lysine or sodium dipicolinate) or with DHDPS which had been inactivated with pyruvate/NaBH₄. Crystals usually grew in 3–5 days for a wide range of conditions, summarized in Table 4.

A new crystal form was, however, obtained in 1.5–2.0 M-ammonium sulphate, pH 4, 22 °C [4 μ l of inactivated enzyme solution 2 mg/ml, 1.5 μ l of precipitating agent and 3 μ l of 5% (w/v) OG in 2 mM-MES, pH 6.8]. These crystals have the shape of small hexagonal rods (< 0.1 mm). In similar conditions, using 1.75 M-sodium/potassium phosphate as precipitant (22 °C, pH 4), very small prisms (< 0.1 mm) were obtained. In 1.5 M-citrate, pH 7.0 [10 mM-L-lysine and 5% (w/v) OG in 2 mM-Mes, pH 6.8, as additives], after transferring the dish from 4 °C to 22 °C, and in 0.5 M-phosphate/10% (w/v) PEG 6000, pH 4.0, at 4 °C (with 10 mM-L-lysine) little prisms were also obtained (< 0.1 mm).

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