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Research paper

How essential is the 'essential' active-site lysine in dihydrodipicolinate synthase?

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ABSTRACT

Dihydrodipicolinate synthase (DHDPS, E.C. 4.2.1.52), a validated antibiotic target, catalyses the first committed step in the lysine biosynthetic pathway: the condensation reaction between (*S*)-aspartate β-semialdehyde [(*S*)-ASA] and pyruvate *via* the formation of a Schiff base intermediate between pyruvate and the absolutely conserved active-site lysine. *Escherichia coli* DHDPS mutants K161A and K161R of the active-site lysine were characterised for the first time. Unexpectedly, the mutant enzymes were still catalytically active, albeit with a significant decrease in activity. The k_{cat} values for DHDPS-K161A and DHDPS-K161R were 0.06 ± 0.02 s^{-1} and 0.16 ± 0.06 s^{-1} respectively, compared to 45 ± 3 s^{-1} for the wild-type enzyme. Remarkably, the K_M values for pyruvate increased by only 3-fold for DHDPS-K161A and DHDPS-K161R (0.45 ± 0.04 mM and 0.57 ± 0.06 mM, compared to 0.15 ± 0.01 mM for the wild-type DHDPS), while the K_M values for (*S*)-ASA remained the same for DHDPS-K161R (0.12 ± 0.01 mM) and increased by only 2-fold for DHDPS-K161A (0.23 ± 0.02 mM) and the K_i for lysine was unchanged. The X-ray crystal structures of DHDPS-K161A and DHDPS-K161R were solved at resolutions of 2.0 and 2.1 Å respectively and showed no changes in their secondary or tertiary structures when compared to the wild-type structure. The crystal structure of DHDPS-K161A with pyruvate bound at the active site was solved at a resolution of 2.3 Å and revealed a defined binding pocket for pyruvate that is thus not dependent upon lysine 161. Taken together with ITC and NMR data, it is concluded that although lysine 161 is important in the wild-type DHDPS-catalysed reaction, it is not absolutely essential for catalysis.

1. Introduction

Dihydrodipicolinate synthase (DHDPS, E.C. 4.2.1.52) is the enzyme that catalyses the first committed step in the lysine biosynthetic pathway and involves the condensation reaction between (*S*)-aspartate β-semialdehyde [(*S*)-ASA] and pyruvate [1,2]. Lysine is an essential amino acid and is part of the aspartate family, including methionine, threonine and isoleucine [3,4]. The biosynthesis of aspartate-derived amino acids is restricted to bacteria, plants and some fungi, so they are essential amino acids in animal nutrition [5]. DHDPS is a validated drug target: Kobayashi et al. [6] identified the *dap*A gene, encoding DHDPS in *Bacillus subtilis*, as one of only 271 genes essential for cell viability from a total of >4100 genes encoded by its genome. DHDPS enzymes from a variety of pathogens have now been characterised in an effort to inform drug discovery programmes [7–10], and studies to unravel the details of the enzymatic mechanism continue.

The accepted mechanism of DHDPS is well characterised [11,12] and is outlined in the Supporting Information (Fig. S1). The first step involves Schiff base formation with pyruvate. (*S*)-ASA then binds to the active site, where it undergoes an aldol-like condensation reaction, followed by the release of the product (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid (HTPA). From the X-ray crystal structure of *Escherichia coli* DHDPS, and alignment of all DHDPS sequences, it was deduced that it is lysine 161 that forms the covalent intermediate with the first substrate, pyruvate [13,14]. This is corroborated by a variety of structural and mass spectrometric studies [15,16]. Remarkably, although lysine161 is accepted as the key active-site residue in DHDPS, no-one has confirmed this using site-directed mutagenesis, presumably because of the expected complete loss of activity.

E. coli DHDPS displays significant sequence and structural homology with respect to *E. coli N*-acetylneuraminic acid lyase (NAL), and these enzymes have remarkably similar quaternary and tertiary

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structures and active sites [17]. When lysine 161 was mutated to an alanine in *Clostridium perfringens* NAL, activity was completely abolished. However, when the residue was mutated to an arginine, 3% of the original activity was maintained [18]. It was speculated that residual activity was present because the guanidinium group of arginine allowed the formation of the Schiff base, but at a slower rate than with the amine group of the active-site lysine, although no evidence was presented in support of these ideas. Equivalent experiments have never been carried out for DHDPS.

The primary aim of this research was to address this gap in our understanding of DHDPS by generating and purifying two site-directed mutants—DHDPS-K161A and DHDPS-K161R—followed by their characterisation using steady-state kinetics, X-ray crystallography, far-UV circular dichroism and isothermal titration calorimetry.

2. Materials and methods

2.1. Materials

Chemicals were purchased (unless otherwise stated) from Sigma–Aldrich (Castle Hill, Australia). Protein concentration was measured using the Bradford assay [19]. Enzymes were manipulated on ice or at 4 °C to reduce denaturation. All enzymes were routinely stored in 20 mM Tris–HCl pH 8.0 at −20 °C in 1 mL aliquots. (*S*)-ASA was prepared *via* the Weinreb method [20]. Stock solutions of (*S*)-ASA and NADPH were prepared fresh for each experiment. DHDPR, the enzyme used in the coupled assay, was purified as previously reported [11,21].

2.2. Site-directed mutagenesis

A QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce the specific changes in the *dap*A gene. The forward primers used were: 5′GGC GAA AGT AAA AAT ATT ATC GGA ATC GCA GAG GCA ACA GGG G3′and 5′GGC GAA AGT AAA AAT ATT ATC GGA ATC AGA GAG GCA ACA GGG G3′ for DHDPS-K161A and DHDPS-K161R respectively; the mutated codon is underlined. The resulting plasmids were sequenced (University of Canterbury Sequencing Facility) to ensure the desired mutation had been introduced and that the integrity of *dap*A was intact after site-directed mutagenesis.

2.3. Over-expression and purification

The purification of DHDPS was based on the methods originally reported by Blickling et al. [14], as modified by Dobson et al. [11]. The crude extract was loaded onto a Q-Sepharose column (bed volume 75 mL, 15 × 2.6 cm) that had been pre-equilibrated with 5 bed volumes of 20 mM Tris–HCl pH 8.0 (buffer A). The column was then washed with 5 bed volumes of the same buffer and the enzyme was eluted with a 0–1 M NaCl gradient in buffer A. Ammonium sulfate was added to the pooled fractions from the ion exchange step to a concentration of 0.5 M. This was loaded onto a phenyl-Sepharose column (bed volume 25 mL, 7 × 2.6 cm) that had been pre-equilibrated with five bed volumes of 0.5 M ammonium sulfate in buffer A. The column was then washed with five bed volumes of the start buffer (0.5 M ammonium sulfate, buffer A) and the enzyme was eluted with an ammonium sulfate gradient (0.5–0 M) in buffer A. The pooled sample from hydrophobic interaction chromatography was further purified by size exclusion chromatography on a Superdex 200 pg column (bed volume 120 mL, 60 × 1.6 cm), eluting with buffer A. *E. coli* DHDPS was over-expressed in *E. coli* AT997recA- that had been transformed with site-directed mutants based on the pBluescript plasmid pJG001. The mutant DHDPS genes were used in place of the wild-type gene in pJG001 during transformation. This strain contains a non-functional chromosomal DHDPS gene (*dap*A$^-$), which was complemented by pJG001 and its mutated derivatives and is recA$^-$ to prevent homologous recombination between a plasmid-borne *dap*A mutant gene and the *E. coli* genome. The strain was grown and maintained in LB in the presence of tetracycline in a medium supplemented with DAP, to allow easy selection and confirmation of the *dap*A$^-$ phenotype. The DHDPS activity from this strain was examined prior to transformation to ensure that the enzyme being produced from the chromosome was indeed non-functional. The ability of transformants to complement the *dap*A$^-$ phenotype was assessed by growing them in the absence of DAP. Each mutant plasmid rescued the AT997recA$^-$ strain, suggesting that they retained sufficient DHDPS activity for survival. Thus the only DHDPS activity in the cells arose from the mutant enzymes.

2.4. Circular dichroism (CD) spectroscopy

Purified wild-type DHDPS, DHDPS-K161A and DHDPS-K161R, which were stored in 20 mM Tris–HCl (pH 8.0), were buffer exchanged into 20 mM NaH_2PO_4, 150 mM NaF, pH 8.0 using a HiTrap desalting column (GE Healthcare Life Sciences) at 4 °C. Final concentrations of the proteins were determined using a NanoDrop ND-1000 (Thermo Fischer Scientific) reading at A_{280} to be ~0.3 mg/mL. CD spectroscopic data were generated using a Jasco CD J-815 circular dichroism spectrophotometer. Spectra were recorded at enzyme concentrations of 9.6 μM. Wavelength scans were collected from 195 to 250 nm in 0.5 nm increments at 20 °C, using a 1 nm bandwidth and a 1 s averaging time in a 1 mm path-length quartz cell. The reported spectra are the average of two scans that were corrected for buffer blanks.

2.5. Steady-state kinetics

DHDPS activity was measured using a coupled assay with DHDPR, as previously described [11,21]. The initial-rate data were collected for the wild-type and mutants in parallel. Typical assays (1 mL) conducted at 30 °C contained 100 mM HEPES, pH 8.0, 0.2 mM NADPH, 50 μg/mL DHDPR and concentrations of (*S*)-ASA and pyruvate varying between 0.04 and 2.5 mM. The assay was initiated by the addition of the enzyme to give final enzyme concentrations of 3.0 μM, 1.7 μM and 0.13 μM for DHDPS-K161A, DHDPS-K161R and wild-type DHDPS respectively. The change in absorbance at 340 nm was recorded over 60 s, blanked against water. Controls with no substrate, and controls lacking DHDPS were also carried out. The initial-rate data collected in duplicate in each of two independent experiments were analysed using the Enzfitter program available from Biosoft (Cambridge, UK). For the mutated enzymes, data were fitted to both tertiary complex and ping-pong mechanisms, but no significant difference in kinetic parameters was obtained. The final kinetic data obtained (Table 1) were fitted

Table 1
Kinetic parameters obtained for DHDPS-K161A and DHDPS-K161R compared with wild-type DHDPS.

Parameter	Wild-type	K161A	K161R
k_{cat} (s^{-1})	45 ± 3	0.06 ± 0.02	0.16 ± 0.06
Relative k_{cat} (%)	100	0.13	0.35
$K_{M,\ Pyruvate}$ (mM)	0.15 ± 0.01	0.45 ± 0.04	0.57 ± 0.06
$K_{M,\ ASA}$ (mM)	0.12 ± 0.01	0.23 ± 0.02	0.12 ± 0.01
$k_{cat}/K_{M,\ Pyruvate}$ ($M^{-1}s^{-1}$)	(3.0 ± 0.4) × 10^5	(1.3 ± 0.6) × 10^2	(2.8 ± 1.3) × 10^2
$k_{cat}/K_{M,\ ASA}$ ($M^{-1}s^{-1}$)	(3.8 ± 0.6) × 10^5	(2.6 ± 1.1) × 10^2	(1.3 ± 0.6) × 10^3
$K_{i,lysine\ wrt\ pyruvate}$ (mM)	0.12 ± 0.01	0.14 ± 0.01	0.14 ± 0.04
$K_{i,lysine\ wrt\ (S)-ASA}$ (mM)	0.18 ± 0.02	0.23 ± 0.04	0.14 ± 0.04