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Analysis of catalytic determinants of diaminopimelate and ornithine decarboxylases using alternate substrates☆

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ABSTRACT

Diaminopimelate decarboxylase (DAPDC) and ornithine decarboxylase (ODC) are pyridoxal 5'-phosphate dependent enzymes that are critical to microbial growth and pathogenicity. The latter is the target of drugs that cure African sleeping sickness, while the former is an attractive target for antibacterials. These two enzymes share the $(\beta/\alpha)_8$ (i.e., TIM barrel) fold with alanine racemase, another pyridoxal 5'-phosphate dependent enzyme critical to bacterial survival. The active site structural homology between DAPDC and ODC is striking even though DAPDC catalyzes the decarboxylation of a D stereocenter with inversion of configuration and ODC catalyzes the decarboxylation of an L stereocenter with retention of configuration. Here, the structural and mechanistic bases of these interesting properties are explored using reactions of alternate substrates with both enzymes. It is concluded that simple binding determinants do not control the observed stereochemical specificities for decarboxylation, and a concerted decarboxylation/proton transfer at Cα of the D stereocenter of diaminopimelate is a possible mechanism for the observed specificity with DAPDC.

1. Introduction

Diaminopimelate decarboxylase (DAPDC) is a pyridoxal phosphate (PLP) dependent enzyme that catalyzes the decarboxylation of D,L-diaminopimelate (D,L-DAP) to form L-lysine in the last step in bacterial L-lysine biosynthesis. The presence of multiple pathways to synthesize D,L-DAP in some bacteria suggests that this pathway is highly important for bacterial survival [1]. Indeed, a functional DAPDC gene is required for *Mycobacterium tuberculosis* survival in immunocompromised mice, demonstrating that *de novo* lysine biosynthesis is essential for in vivo viability [2]. Combined with the absence of analogous pathways in humans, this makes DAPDC an attractive antibacterial drug target.

Scheme 1

Mechanistically, DAPDC has unique features and challenges. First, it must differentiate between the D and L stereocenters of D,L-DAP, selectively decarboxylating only the D stereocenter to generate L-lysine. X-ray crystallographic studies of DAPDC from *M. tuberculosis* (*mt*DAPDC), as well as other bacteria, suggest a structural basis for this specificity, especially through specific binding of the L stereocenter distal to the reaction center [2,3]. Second, DAPDC is unique among known PLP-dependent decarboxylases in decarboxylating a D stereocenter; all other PLP-dependent decarboxylases act on an L stereocenter. Third, stereochemical studies show that DAPDC catalyzes decarboxylation with inversion of configuration at the reaction center [4,5], in contrast to all other PLP-dependent decarboxylases studied to date [6–11].

Despite these differences, DAPDC shares both sequence and structural similarity to eukaryotic ornithine decarboxylase (ODC), a typical PLP-dependent decarboxylase. They have 25% sequence identity [3], and structural overlays of *mt*DAPDC and *Trypanosoma brucei* ODC have an rmsd of ~2.2 Å [12]. Furthermore, the active sites of *mt*DAPDC and ODC have many structural equivalences, including the KAFL motif that contains the lysine that forms the internal aldimine with PLP and a glutamate residue that interacts with the pyridine nitrogen of PLP. In addition, the His that π stacks with the PLP ring is conserved and similarities in the side chain binding pocket including a conserved arginine and serine. The active site structural similarity between *mt*DAPDC and ODC implies a similar chemical mechanism despite the stereochemical differences between the enzymes. Here, kinetic characterization of *mt*DAPDC and yeast ODC using alternate substrates is used to explore the strictness of DAPDC specificity for decarboxylating a D stereocenter, as well as the origin of this stereospecificity.

2. Experimental procedures

2.1. Materials

All materials were purchased from Sigma unless otherwise noted. D,L-DAP, L,L-DAP and D,D-DAP were a gift from Professor John Blanchard (Albert Einstein College of Medicine).

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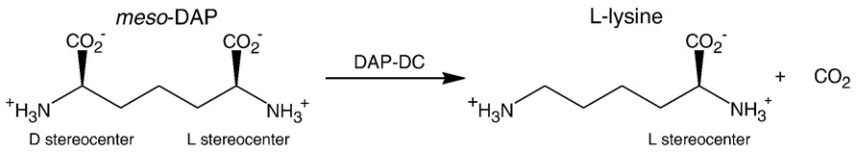

Scheme 1. Reaction catalyzed by DAPDC.

2.2. Cloning of yeast ODC gene

The yeast ODC gene was amplified from genomic DNA from the WDHT668 strain of *Saccharomyces cerevisiae* using PCR and the following primer pair: 5′-GTG GTG CTC GAG TCA ATC GAG TTC AGA GTC TAT GTA TAC TAT ATC C-3′ (XhoI restriction site is underlined) and 5′-GGA TCC GAA TTC ATG TCT AGT ACT CAA GTA GGA AAT GCT CTA TCT AG-3′ (EcoRI restriction site is underlined). The ~1.4 kb PCR product was cleaned using a commercially available kit (Qiagen) and ligated into pET28a (Novagen) digested with XhoI and EcoRI using standard procedures [13]. The construct was sequenced to ensure no mutations had been incorporated during PCR and yielded the final product, an N-terminal His_6-tagged yeast ODC fusion protein.

2.3. Expression and purification of yeast ODC

For overexpression, 1 l of LB growth medium was innoculated with a 5 ml overnight culture and grown at 37 °C until $OD_{600} = 0.5$. Expression was induced with IPTG at a final concentration of 500 μM, and growth at 37 °C was continued for 6–8 h. The cells were pelleted by centrifugation, resuspended in lysis buffer (20 mM TEA–HCl pH 7.8, 10 mM imidazole, 50 μM PLP and 1 mM β-mercaptoethanol) and stored overnight at −80 °C. The thawed cell suspension was incubated with 1 mg/ml lysozyme for 1 h, sonicated and the cell debris removed by centrifugation. The cell free extract was added to 8 ml Ni-NTA Superflow resin (Qiagen) and mixed by end-to-end rotation for 1 h at 4 °C. After packing, the column was washed extensively (>20 column volumes) with 20 mM TEA–HCl pH 7.8, 20 mM imidazole, 50 μM PLP, 1 mM β-mercaptoethanol and 300 mM KCl. The protein was eluted by running a linear gradient from 20 mM imidazole to 300 mM imidazole in 20 mM TEA–HCl pH 7.8, 50 μM PLP, 1 mM β-mercaptoethanol and 300 mM KCl at 1 ml/min. The fractions containing ODC, as judged by SDS-PAGE, were pooled, concentrated and dialyzed into 50 mM TEA–HCl pH 7.8, 50 mM KCl, 20 μM PLP and 1 mM DTT. The protein was aliquoted, flash frozen and stored at −80 °C. The enzyme concentration was determined using the Lowry Assay (BioRad) with IgG as a standard.

2.4. Expression and purification of M. tuberculosis DAPDC

For overexpression, 1 l LB growth medium was innoculated with 3 ml overnight culture and grown at 37 °C until $OD_{600} = 0.5$. The cells were placed on ice for 30 min, induced with IPTG at a final concentration of 500 μM, and grown at 20 °C for 24 h. The cells were pelleted by centrifugation, resuspended in lysis buffer (20 mM TEA–HCl pH 7.8, 10 mM imidazole, 50 μM PLP and 1 mM β-mercaptoethanol) and stored overnight at −80 °C. The thawed cell suspension was incubated with 0.5 mg/ml lysozyme for 1 h, sonicated and the cell debris removed by centrifugation. The cell free extract was added to 8 ml Ni-NTA Superflow resin (Qiagen), and purified as described above for yeast ODC. The protein was aliquoted, flash frozen and stored at −80 °C. The enzyme concentration was determined using the Bradford and Lowry assays (BioRad) with IgG as a standard.

2.5. Steady-state assay

Yeast ODC and *mt*DAPDC were assayed as described previously [14,15] by coupling the carbon dioxide produced from decarboxylation to phosphoenol pyruvate (PEP) carboxylase to give oxalacetate, followed by malate dehydrogenase (MDH) catalyzed reduction. Loss of NADH absorbance at 340 nm was followed on a Kontron UVIKON 9420. Reaction mixtures contained 100 mM TEA–HCl pH 7.8, 20 μM PLP, 300 μM NADH, 10 mM $MgCl_2$, 2 mM PEP, 8 units/ml freshly prepared PEP carboxylase and 33 units/ml MDH. All reagents were prepared in water that had been boiled extensively to remove carbon dioxide. Stock solutions were lyophilized and redissolved in water from which the carbon dioxide had been removed.

2.6. Inhibition assays

Inhibition studies for ODC were performed with D-ornithine and putrescine, using the coupled carbon dioxide assay. The concentration of L-ornithine was held at K_m (130 μM) and K_i was determined assuming competitive inhibition (Eq.1).

$$v_i = \frac{V_{max}[S]}{K_m\left(1 + {}^{[I]}/_{K_i}\right) + [S]} \quad (1)$$

Inhibition studies for *mt*DAPDC used L- and D-lysine, L,L- and D,D-DAP using the coupled carbon dioxide assay. The concentration of D,L-DAP was held at K_m (400 μM) and K_i was determined assuming competitive inhibition.

2.7. Circular dichroism-based assay

For both ODC and DAPDC, the substrate and product differ in their optical activity, allowing direct detection of the reaction using circular dichroism (CD). A Jasco J-600 CD spectrometer was used to assay ODC activity, as previously reported for *T. brucei* ODC [16]. The loss of CD signal at 210 nm was monitored over time. The wavelength was chosen to maximize signal while maintaining a linear relationship between CD signal and amino acid substrate concentration. Reaction mixtures contained 20 mM potassium phosphate pH 7.5, 20 μM PLP, and 100 mM KCl.

2.8. HPLC assay for reaction with alternate substrates

Assays to identify the amine or amino acid product of decarboxylation were performed using a previously reported HPLC-based method [17]. Reactions with 100 mM TEA–HCl pH 7.8, 100 mM KCl, 20 μM PLP, and the appropriate alternate substrate and enzyme were allowed to react at 25 °C for 12–16 h. The protein was denatured by adding 2 μl glacial acetic acid to the 200 μl reaction and the precipitated protein was removed by centrifugation. The reaction mixture was then derivatized with *o*-phthaldialdehyde (OPA). Briefly, a 100 μl sample was mixed with 100 μl freshly prepared OPA reagent (4 mg solid OPA, mixed with 4.5 ml 0.1 M borate pH 10.4, 15 μl 30% (w/v) Brij detergent and 10 μl β-mercaptoethanol), allowed to react for 1 min at room temperature and acidified with 2 M acetic acid to pH 4.5. The derivatized sample was immediately run on a Hibar LiChrosorb C18 column using an Agilent 1100 series chromatography system attached to a Perkin-Elmer 650-15 fluorescence spectrophotometer. The HPLC method [17] used 50 mM sodium acetate pH 5.7, 5% (v/v) THF (Solvent A); methanol (Solvent B); acetonitrile (Solvent