Biochimica et Biophysica Acta 1854 (2015) 146–155

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

# Directed evolution of the substrate specificity of dialkylglycine decarboxylase☆

Jared L. Taylor, Joseph E. Price, Michael D. Toney *

*Department of Chemistry, University of California, Davis, CA 95616, USA*

ARTICLE INFO

*Article history:*
Received 26 August 2014
Received in revised form 19 November 2014
Accepted 3 December 2014
Available online 10 December 2014

*Keywords:*
Pyridoxal phosphate
Dialkylglycine decarboxylase
Directed evolution
Genetic selection
Conformational change
Stereoelectronic effect

ABSTRACT

Dialkylglycine decarboxylase (DGD) is an unusual pyridoxal phosphate dependent enzyme that catalyzes decarboxylation in the first and transamination in the second half-reaction of its ping-pong catalytic cycle. Directed evolution was employed to alter the substrate specificity of DGD from 2-aminoisobutyrate (AIB) to 1-aminocyclohexane-1-carboxylate (AC6C). Four rounds of directed evolution led to the identification of several mutants, with clones in the final rounds containing five persistent mutations. The best clones show ~2.5-fold decrease in $K_M$ and ~2-fold increase in $k_{cat}$, giving a modest ~5-fold increase in catalytic efficiency for AC6C. Additional rounds of directed evolution did not improve catalytic activity toward AC6C. Only one (S306F) of the five persistent mutations is close to the active site. S306F was observed in all 33 clones except one, and the mutation is shown to stabilize the enzyme toward denaturation. The other four persistent mutations are near the surface of the enzyme. The S306F mutation and the distal mutations all have significant effects on the kinetic parameters for AIB and AC6C. Molecular dynamics simulations suggest that the mutations alter the conformational landscape of the enzyme, favoring a more open active site conformation that facilitates the reactivity of the larger substrate. We speculate that the small increases in $k_{cat}/K_M$ for AC6C are due to two constraints. The first is the mechanistic requirement for catalyzing oxidative decarboxylation via a concerted decarboxylation/proton transfer transition state. The second is that DGD must catalyze transamination at the same active site in the second half-reaction of the ping-pong catalytic cycle.



## 1. Introduction

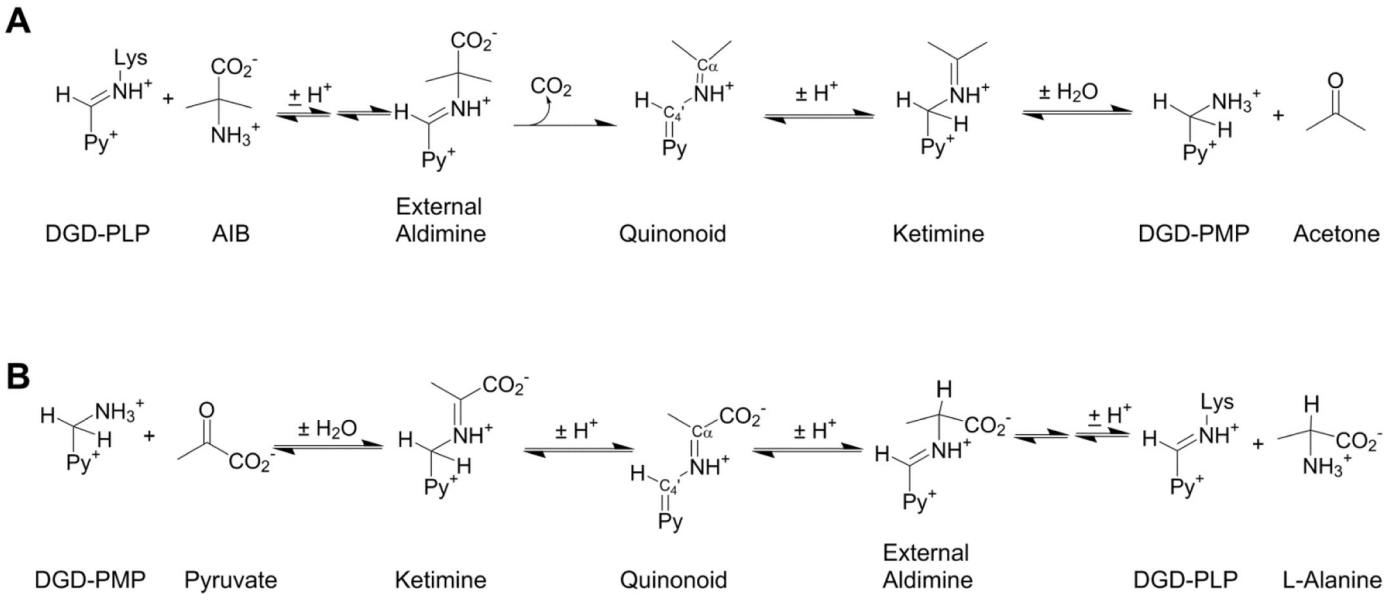
Dialkylglycine decarboxylase (DGD) is a pyridoxal phosphate (PLP) dependent enzyme, first isolated from the soil bacterium *Pseudomomas cepacia* [1,2]. It catalyzes two different reactions – decarboxylation-dependent transamination of 2-aminoisobutyrate (AIB; α-methylalanine) to generate the pyridoxamine phosphate enzyme, followed by transamination of pyruvate to L-alanine to regenerate the PLP enzyme – at the same active site. In the first half-reaction of the ping-pong kinetic mechanism (Scheme 1A), DGD catalyzes decarboxylation of AIB to form $CO_2$ and acetone with the amino group of AIB transferred to the cofactor to form pyridoxamine phosphate. In the second half-reaction (Scheme 1B), the amino group is transferred to an α-keto acid (pyruvate, the preferred in vitro substrate and presumed in vivo substrate, is shown) to form an L-amino acid product and regenerate the PLP cofactor.

It is fundamentally important to understand the mechanisms by which reaction and substrate specificity are controlled by PLP-dependent enzymes, given their broad role in the nitrogen metabolism of all organisms [3–9]. DGD is especially interesting in this context because of its unusual dual (decarboxylation and transamination) reaction specificity. A functional active site model for DGD was proposed by Toney et al. [10] based on the X-ray structure of DGD and previous kinetic studies [11]. In this model (Fig. 1), the active site of DGD is described by three binding subsites (A, B, and C), each of which differs in specificity and function. The A subsite is the locus of bond making and breaking for both decarboxylation and transamination. The B subsite is capable of binding both aliphatic groups (AIB in decarboxylation), or carboxylate groups (pyruvate in transamination). The C subsite is sterically restrictive, binding small alkyl groups. Catalytic efficiency decreases sharply as larger side chains occupy the C subsite [11]. This steric restriction is thought to be responsible for the poor efficiency of DGD with bulky substrates such as 1-amino-1-cyclopentanecarboxylic acid (AC5C) and 1-amino-1-cyclohexanecarboxylic acid (AC6C) (Fig. 2) [11]. The C subsite reduces the binding affinity for AC5C and AC6C and their bound forms are thought to be misaligned for optimal catalysis thereby diminishing favorable stereoelectronic effects (Fig. 1).

The primary means by which the PLP-enzymes control reaction specificity is stereoelectronic [8,9,12]. Stereoelectronic effects are maximized when the labile bond is parallel to the *p* orbitals of the conjugated π system (Schiff base and pyridine ring). This, in turn, maximizes the rate of bond scission by stabilizing developing negative charge in the

☆ This work was supported by grant GM54779 from the National Institutes of Health.
* Corresponding author. Tel.: +1 530 754 5282.
*E-mail address:* mdtoney@ucdavis.edu (M.D. Toney).

http://dx.doi.org/10.1016/j.bbapap.2014.12.003
1570-9639/




**Scheme 1.** Half-reactions of the DGD catalyzed ping-pong mechanism. The enzyme avoids the carbanionic quinonoid intermediate in the decarboxylation half-reaction by enforcing a concerted decarboxylation/proton transfer transition state between external aldimine and ketimine intermediates.

transition state through delocalization into the conjugated π system of PLP. Evidence for stereoelectronic effects controlling DGD catalysis includes kinetic studies with alternate substrates [11], X-ray crystallography studies with phosphonate inhibitors [13], and studies performed with DGD mutants [14,15].

Here, we report directed evolution of DGD substrate specificity to examine the ability of the catalytically bifunctional active site to adapt to a new, larger substrate in the decarboxylation half-reaction while maintaining specificity in the transamination half-reaction for pyruvate, the presumed in vivo substrate. A synthetic gene optimized for *Escherichia coli* expression was constructed. Error-prone PCR and gene shuffling mutagenesis techniques were combined with a genetic selection to isolate DGD mutants with altered specificity for the non-native substrate AC6C. Several mutants with increasing catalytic efficiency were purified and analyzed kinetically. The best mutants isolated show a modest ~5-fold increase in $k_{cat}/K_M$ for AC6C compared to the WT enzyme. This modest increase is interpreted as a result of the requirement for a concerted decarboxylation/proton transfer transition state to enforce oxidative decarboxylation, and the requirement for maintaining high efficiency for the small substrate pyruvate in the transamination half-reaction.

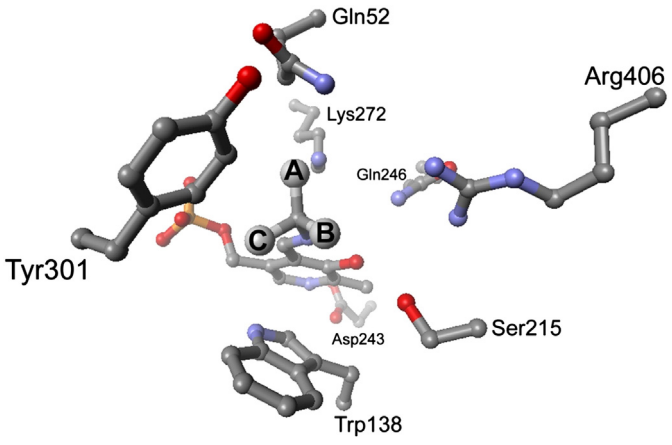



**Fig. 1.** Model of the DGD active site. The three subsites discussed in the text are labeled as A, B, and C. The alignment of the Cα-$CO_2^-$ bond with the *p* orbitals of the conjugated π system (Schiff base and pyridine ring) when it is in the A subsite stabilizes negative charge as it develops on Cα in the transition state. This is the stereoelectronic effect referred to in the text.

## 2. Materials and methods

### 2.1. Materials

Oligonucleotides were obtained from Invitrogen. The Pt-*Taq* polymerase was from Invitrogen. A 2.5 mM dNTP PCR mix was from Applied Biosystems. *Pfu*Turbo polymerase and the *E. coli* BL21(DE3)-Gold cells were from Stratagene. The pProEX-HtaB plasmid was from Life Technologies. The pBTac plasmid was from Professor John Keller (Department of Chemistry, University of Alaska) [16]. DNA purification kits were from QIAGEN. Centri-spin 20 size exclusion columns were from Princeton Separations. T4 DNA ligase and all restriction endonucleases were from New England Biolabs. AIB, sodium pyruvate and all antibiotics were from Sigma-Aldrich. AC6C and AC5C were from Acros. NADPH was from Roche. All other media components were from Fisher Scientific. All PCR was carried out using or an Applied Biosystems GeneAmp PCR System 2700 thermocycler. Bacterial transformations were carried out using a Bio-Rad Micropulser electroporation apparatus.

### 2.2. Gene synthesis

The sequence of a synthetic *dgdA* gene, designated *dgdAecx* ("ecx" for *E. coli* expression), was designed using only the preferred codon usage for strongly expressed *E. coli* genes [17]. The DGD amino acid sequence (taken from PDB entry 1DKA) was reverse translated into the synthetic gene using a table of codons that contained a single entry for each amino acid type. The entry for each was the most frequently employed codon in highly expressed *E. coli* genes. The resulting

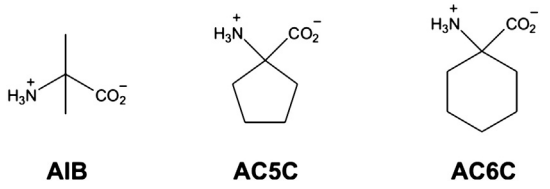



**Fig. 2.** DGD substrates. AIB is the natural substrate of DGD and AC6C was the target substrate here.