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# Studies of lysine cyclodeaminase from *Streptomyces pristinaespiralis*: Insights into the complex transition $NAD^+$ state

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ABSTRACT

Lysine cyclodeaminase (LCD) catalyzes the piperidine ring formation in macrolide-pipecolate natural products metabolic pathways from a lysine substrate through a combination of cyclization and deamination. This enzyme belongs to a unique enzyme class, which uses $NAD^+$ as the catalytic prosthetic group instead of as the co-substrate. To understand the molecular details of $NAD^+$ functions in lysine cyclodeaminase, we have determined four ternary crystal structure complexes of LCD-$NAD^+$ with pipecolic acid (LCD-PA), lysine (LCD-LYS), and an intermediate (LCD-INT) as ligands at 2.26-, 2.00-, 2.17- and 1.80 Å resolutions, respectively. By combining computational studies, a $NAD^+$-mediated "gate keeper" function involving $NAD^+$/NADH and Arg49 that control the binding and entry of the ligand lysine was revealed, confirming the critical roles of $NAD^+$ in the substrate access process. Further, in the gate opening form, a substrate delivery tunnel between ε-carboxyl moiety of Glu264 and the α-carboxyl moiety of Asp236 was observed through a comparison of four structure complexes. The LCD structure details including $NAD^+$-mediated "gate keeper" and substrate tunnel may assist in the exploration the $NAD^+$ function in this unique enzyme class, and in regulation of macrolide-pipecolate natural product synthesis.



## 1. Introduction

In most nicotinamide dinucleotide-dependent reactions, $NAD(P)^+$ is in essence a co-substrate; however, there exists a class of enzymatic reactions in which the coenzyme is tightly bound and acts as a catalytic prosthetic group [1]. Catalytic usage of $NAD^+$, referred as "complex $NAD^+$-dependent transformation", has been proposed in a number of vital cellular enzymes such as: myo-inositol-1-phosphate synthase [2], UDP-galactose epimerase [3], ornithine cyclodeaminase (OCD) and lysine cyclodeaminase (LCD) [4–6]. A characteristic of the mechanisms of these enzymes is that the nicotinamide cofactor could be recycled to its original oxidation state after the catalytic reaction finished [7].

LCD catalyzes the rate-limiting step of piperidine formation in macrolide-pipecolate natural products metabolic pathways from a lysine substrate through a combination of cyclization and deamination (Fig. 1) [8]. While little is known about the properties of the LCD structure and enzymatic process, there have been several published reports on the identification and characterization of OCD enzymes [9,10]. OCD was found to performs a cyclization and deamination reaction to convert ornithine directly to proline [9]. Costilow et al. first described the "cyclodeamination" activity of OCD purified from *Clostridium sporogenes*, they noted that the α-amino group, as opposed to that at the ε-position, is lost during the reaction [10]. The essential cyclideamination reaction process of OCD and LCD is composed of an oxidation, a rearrangement and a reduction which did not result in a net change in the oxidation state between substrate and products as well as the nicotinamide co-factors. The unusual overall cryptic redox cyclodeamination process and other properties of LCD may partially owing to its unique catalytic usage of $NAD^+$ [8].

Recently, Walsh et al. and Tsotsou et al. characterized the basic biochemical properties of LCD and suggested that the overall catalytic reaction to convert lysine into pipecolic acid may be akin to

*Abbreviations:* LCD, lysine cyclodeaminase; OCD, ornithine cyclodeaminase; LYS, L-lysine; PA, L-pipecolic acid; INT, intermediate.

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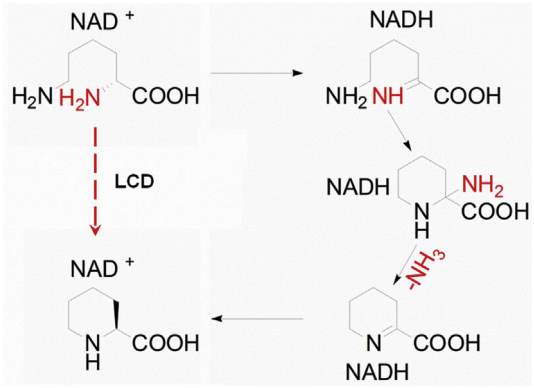


**Fig. 1.** Description of the enzymatic process of the lysine cyclodeaminase (LCD).

OCD [8,11]. However, the exact functions of the nicotinamide cofactor during the enzymatic process remain unclear. Here, we report the first structure determination of LCD and three ternary complexes. By combining molecular dynamic (MD) simulations, an $NAD^+$-mediated "gate keeper" structure on the LCD surface at an exterior position facing the buried active site which controls substrate binding and a substrate delivery tunnel was identified. Understanding these structures and processes should help clarify the mechanisms of "complex $NAD^+$-dependent transformation" in this unique enzyme class.

## 2. Materials and methods

### 2.1. Protein expression, purification and activity assays

We used previously-established protocols for the expression, purification of LCD, as well as the kinetics and enzyme activity assays [8]. *PipA* gene encoding lysine cyclodeaminase from *Streptomyces pristinaespiralis* ATCC 25486 is artificially synthesized by Genescript. co, Nanjing Jiangsu. *Escherichia coli* strain BL21 (DE3) cells harboring the pET28b-*pipA* plasmid were grown at 37 °C. N-terminally His6-tagged LCD was over-expressed following induction with 100 μM isopropyl-β-D- 1-thioga-lactopyranoside for 18 h at 25 °C. After cell lysis, LCD was purified via immobilized $Ni^{2+}$ affinity chromatography (His-Trap HP; GE Healthcare), followed by both anion exchange chromatography (Resource Q; GE Healthcare) and size-exclusion chromatography (Superdex 200 10/300 GL; GE Healthcare). Purified LCD (in 50 mM HEPES-NaOH buffer, pH 7.0, HEPES-NaOH buffer was prepared by dissolving the HEPES into the distilled water and adjusted to pH 7.0 using 1 M NaOH) was concentrated to 10 mg/ml using an Amicon Ultra-430K filter (Millipore) and stored frozen at −80 °C prior to use. All chromatography protocols were performed using an ÄKTA-explorer apparatus (GE Healthcare). Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as the standard. Protein purity was monitored by SDS-PAGE.

To determine the catalytic activity, L-lysine (6.8 mM, 1.0 mg/mL) was incubated in a final volume of 1 mL with 0.012 mM (0.5 mg/mL) LCD, 50 mM $NAD^+$, 50 mM HEPES (pH 7.0) at 37 °C for 4h. Reactions were run in triplicate and initiated by the addition of LCD. After the reactions were finished, the reaction mixtures were centrifugated and the supernatant were harvest and stored in −80 °C for further analysis.

### 2.2. Protein crystallization, data collection and structure determination

LCD was crystallized using the sitting drop method at room temperature. 0.30 μl drops containing 0.15 μL of protein sample (10 mg/ml protein) mixed with 0.15 μl of reservoir solution (2% (v/v) polyethylene glycol 400, 0.1 M HEPES) were equilibrated against wells containing 50 μl of the reservoir solution. Rectangular crystals grew to maximal dimensions in 5–7 days.

A reservoir solution (to which had been added an extra 25% v/v glycerol as a cryo-protectant) was used to flash freeze the well-ordered single crystals. These were then mounted on the goniometer of a home source diffraction system (Rigaku) at 100K. All of the data were collected with a Rigaku F-RE++ superbright generator, with either a CCD Saturn 944HG detector or a RAXIS IV IP detector. HKL3000 software was used to merge and scale the data.

To solve the initial phase of LCD, the single-wavelength anomalous diffraction (SAD) experimental method was used. A 0.5 M $KI_3$ solution (0.5 M KI plus 0.5 M $I_2$) was prepared in water and further diluted to 50 mM with cryo-protectant solution for use as the $KI_3$ soaking solution. 0.5 μl of this $KI_3$ soaking solution was added to the sitting well near the crystallization drops with single crystals, and the $KI_3$ and crystals were allowed to mix for about 10 min. These were then flash frozen and mounted on the goniometer of the diffraction instrument, and high redundancy data was collected with the CCD Saturn 944HG detector.

To obtain different soaks, crystals LCD-$NAD^+$ were transferred to mother liquor solution containing ~1 mM/L-lysine and incubated for 1–120 min before flash freezing in liquid nitrogen.

### 2.3. MD simulations, free energy calculation

Models of LCD-$NAD^+$ and LCD-NADH were constructed as following. First, optimization was performed with the Gaussian 09 program at the level of HF/6-31G(d) [12–14]. This was followed by calculation of the electrostatic surface potential (ESP) charges. A restrained electrostatic potential (RESP) [15] charge fitting was applied. Finally, using the AMBER suite (with ff03.r1 force field), MD simulations were conducted with the modeled structures of the four systems. All ionizable side chains were maintained in their standard protonation states at pH 7.0. The proteins were solvated in a cubic box of TIP3P water molecules, with a water thickness extending at least 10 Å away from the protein surface. Sodium ions were then added to the system as counter ions to create a neutral simulation system.

In an attempt to preclude instability that might occur during the MD simulations, the solvated system was subjected to 10,000 steps of minimization, and was changed from the steepest descent algorithm to a conjugate gradient algorithm after 1000 cycles. The system was then gradually heated from 0 K to 300 K in 50 ps steps controlled by Langevin dynamics with a collision frequency of 2 $ps^{-1}$. Next, the system was switched to constant pressure and temperature (NPT) and was equilibrated for 50 ps to facilitate the adjustment of the system to the appropriate density. Finally, the product simulations were carried out in the absence of any restraints under NPT conditions, followed by a 100 ns MD simulation. Each simulation was repeated 2 times. The Particle Mesh Ewald (PME) method [16] was used to calculate long-range electrostatic interactions. The lengths of bonds involved in hydrogen atoms were fixed with the SHAKE algorithm [17]. During the simulations, an integration time step of 2 fs was adopted, and structural snapshots were flushed every 500 steps (1ps). The non-bonded cutoff was set to 10.0 Å. This protocol was applied to all of the simulation systems.

MM-PBSA method implemented in the AMBER12 software suite was used to calculate the free energies ($\Delta G$) in solution between LCD-$NAD^+$ and LCD-NADH. The MM-PBSA calculation process can be conceptually summarized as:

$$\Delta G = G_{cis} - G_{trans} \quad (1)$$

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