Plant Science 271 (2018) 133–142

Contents lists available at ScienceDirect

Plant Science

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

Characterization of L-aspartate oxidase from *Arabidopsis thaliana*☆

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ARTICLE INFO

Keywords:
L-aspartate oxidase
Arabidopsis thaliana
NAD^+ biosynthesis
Regulation
Competitive inhibition

ABSTRACT

The flavoprotein L-aspartate oxidase (LASPO) is the first enzyme of the *de novo* biosynthetic pathway of NAD^+ in plants. Although LASPO is considered pivotal to maintain NAD^+ homeostasis, it has not been hitherto characterized in plants. Here, the cDNA encoding the LASPO from the model plant *Arabidopsis thaliana* (*AtLASPO*, *At5g14760*) has been cloned and expressed in *Escherichia coli* for subsequent enzyme characterization. The purified *At*LASPO enzyme displayed a K_m of 0.79 mM for L-aspartate and a k_{cat} of 0.25 s^{-1}. We could further detect an L-aspartate: fumarate oxidoreductase activity of the recombinant plant enzyme. In addition, results indicated that $NADP^+$ but not NAD^+, and even more strongly NADH, inhibited *At*LASPO at physiological concentrations by competing with the flavin for binding to the apoprotein. LASPO optimal pH and temperature, as well as plastidial pyridine nucleotide concentrations may contribute to an increased NAD^+ production *in planta*. Moreover, in *Arabidopsis thaliana AtLASPO* gene expression exhibited a clear correlation between LASPO activity and NAD^+ levels, thus demonstrating that plant LASPO catalyzes a key metabolic step of NAD^+ synthesis.

1. Introduction

NAD^+ is a ubiquitous coenzyme in reduction-oxidation (redox) reactions in all living organisms, and recent data suggest that it has new roles as a signaling molecule in several stress response mechanisms [1–3]. In plants and most bacteria, NAD^+ is produced by a *de novo* pathway starting from L-aspartate as a precursor. In the model plant *Arabidopsis thaliana*, the first three steps of the *de novo* NAD^+ biosynthetic pathway are plastidial and catalyzed by L-aspartate oxidase (LASPO; EC 1.4.3.16), quinolinate synthetase (QS; EC 2.5.1.72) and quinolinate phosphoribosyltransferase (QPT; EC 2.4.2.19), respectively [4]. The final steps are performed in the cytosol by nicotinate mononucleotide adenylyltransferase (NaMNAT; EC 2.7.7.18) and NAD synthetase (NADS; EC 6.3.5.1) (Fig. 1A) [5–7].

LASPO is a flavoprotein containing 1 mol of non-covalently bound FAD per mol of protein. In bacteria, LASPO is encoded by *nadB* and it is a key regulatory enzyme of NAD^+ biosynthesis and subjected to feedback inhibition by NAD^+ [8,9]. LASPO has been purified from *E. coli* (*Ec*LASPO) [10], *Pyrococcu shorikishii* OT-3 (*Ph*LASPO) [11], *Sulfolobus tokodaii* (*St*LASPO) [12,13], *Bacillus subtilis* (*Bs*LASPO) [14] and *Pseudomonas putida* (*Pp*LASPO) [15]. Crystallization of *Ec*LASPO coupled with site-directed mutagenesis allowed the identification of amino acid residues involved in the FAD- and succinate-binding sites [16–18]. These amino acids are strictly conserved between plant and bacterial species [19]. *Ec*LASPO is a member of the succinate dehydrogenase/fumarate reductase family of enzymes [16] and, atypically, it can use both oxygen and fumarate as electron acceptors in the L-aspartate oxidation reaction [20] (Fig. 1B). Hence, the enzyme shows not only L-aspartate oxidase activity but also fumarate reductase and L-aspartate: fumarate oxidoreductase activities [20]. In addition, *St*LASPO and

Abbreviations: DE, dicarboxylic exchangers; FAD, flavin adenine dinucleotide; $FADH_2$, FAD hydroquinone form; GST, glutathione *S*-transferase; iminoasp., iminoaspartate; IPTG, isopropyl thio-β-D-galactoside; LASPO, L-aspartate oxidase; NAD^+, nicotinamide adenine dinucleotide; NADS, NAD synthetase; NaMNAT, nicotinate mononucleotide adenylyltransferase; PeT, chloroplastic electron transfer chain; PMSF, phenylmethanesulfonyl fluoride; QPT, quinolinate phosphoribosyltransferase; QS, quinolinate synthetase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

☆ This work was supported by the University Paris-Sud, a fellowship from the China Scholarship Council (CSC) and a public grant overseen by the French National Research Agency (ANR) as part of the « Investissement d'Avenir» program, through the "Lidex-3P" project and a French State grant (ANR-10-LABX-0040-SPS) funded by the IDEX Paris-Saclay, ANR-11-IDEX-0003-02. LdB was supported by the French National Research Agency (ANR-2010-GENOM-BTV-005-01) « EFG-MIG» project.

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https://doi.org/10.1016/j.plantsci.2018.03.016
Received 15 February 2018; Received in revised form 15 March 2018; Accepted 16 March 2018
Available online 19 March 2018
0168-9452/

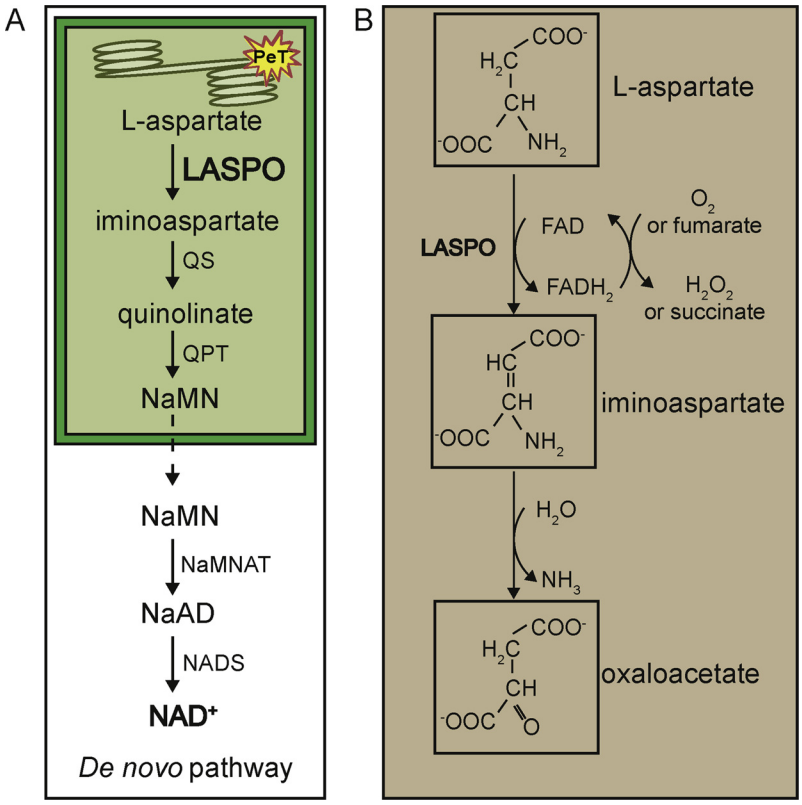

Fig. 1. *De novo* pathway of NAD^+ in *Arabidopsis thaliana* and the reactions catalyzed by LASPO. (A) Five steps are carried out in NAD^+ biosynthesis. Nicotinate mononucleotide (NaMN) is formed from L-aspartate in chloroplast (PeT, chloroplastic electron transfer chain) in the first three steps catalyzed by LASPO, quinolinate synthetase (QS) and quinolinate phosphoribosyltransferase (QPT), in which LASPO, as the first enzyme, catalyzes the oxidation of L-aspartate. NaMN is converted to NAD^+ in two steps catalyzed by nicotinate mononucleotide adenylyltransferase (NaMNAT) and NAD synthetase (NADS). (B) LASPO can use both oxygen and fumarate as electron acceptors for FAD reoxidation, and produce iminoasparate. Iminoaspartate is unstable and nonenzymatically hydrolyzed to oxaloacetate.

*Pp*LASPO display an activity on L-asparagine [13,15].

To date, the biochemical properties of the plant LASPO are unknown. Although it was reported that LASPO activity could be detected in cotton callus cell extracts [21], it was revealed later that this report was erroneous [22]. In the model plant *Arabidopsis thaliana* (Arabidopsis), the single *FIN4* gene (*At5g14760*) encodes a plastidial LASPO (*At*LASPO) required for normal growth and development [4]. Recently, three *FIN4* T-DNA insertion mutants (named *fin4-1; fin4-2, SALK_013920C;* and *fin4-3, SAIL_1145_B10*) were identified [4,23] and both *fin4-1* and *fin4-3* harbored impaired stomatal immunity against the hemibiotrophic bacterial plant pathogen *Pseudomonas syringae* [23]. In addition, we previously reported an up-regulation of *At*LASPO transcripts in Arabidopsis leaves infected with an avirulent strain of *Pseudomonas syringae* [24]. Hence, this suggests an important role for *At*LASPO in NAD^+ production under pathogen stress [3]. However, our knowledge is still fragmentary with respect to the biochemical properties of plant LASPO. Given the growing amount of evidence concerning the importance of NAD^+ homeostasis in plant productivity and stress resistance [25], it thus appeared critical to gain a thorough knowledge of the biochemical properties of plant LASPO.

To investigate the role of LASPO in plant NAD^+ biosynthesis, we determined LASPO biochemical properties by cloning and expressing *At*LASPO in *E. coli*. The recombinant enzyme was used to determine pH and temperature optima and the kinetic parameters of *At*LAPSO. As for *E. coli,* an L-aspartate: fumarate oxidoreductase activity for *At*LASPO was detected. However, whilst strong sequence similarities suggested shared biochemical functions between plant and bacterial enzymes, *At*LASPO was unable to catalyze fumarate reductase activity in the absence of L-aspartate and it lacked inhibition by NAD^+ at physiological NAD^+ concentrations. In contrast, NADH, and to a lesser extent $NADP^+$, inhibited the plant apoenzyme. This work is the first characterization of a plant LASPO and thus helps broaden our understanding of NAD^+ biosynthesis in higher plants. Additionally, we also find that, in *Arabidopsis thaliana*, the loss of *At*LASPO activity decreased NAD^+ levels and in contrast, an overexpression of *At*LASPO increased NAD^+ contents, strongly suggesting that *At*LASPO is limiting for NAD^+ production in Arabidopsis.

2. Materials and methods

2.1. AtLASPO cloning and expression in E. coli

The *AtLASPO* coding sequence was amplified from a full-length *AtLASPO* cDNA obtained from the *Arabidopsis thaliana* Biological Resource Center (stock number G12956) using primers 5′-CCGGAATT CGCTGTTTCGGTATCTTCTTC-3′(with an added *Eco*R1 restriction site) and 5′-ATCCGCTCGAGGCAATCAATAAGTGAGCT-3′(containing an added *Xho*1 restriction site). The PCR-amplified sequence excluded 222 nucleic acids from the initiation codon which encoded the chloroplast transit peptide. The PCR fragment was recombined into pGEX-4T-1 to allow the production of an N-terminal GST-tagged AtLASPO protein. The resulting expression plasmid was used to transform *E. coli* BL21 (DE3) cells. Recombinant protein production was induced by the addition of 1 mM IPTG to pGEX-4T-1-AtLAPSO transformed *E. coli* cells grown to an OD of 0.6 at 37 °C followed by 6 h of culture at 28 °C. Cells were harvested by centrifugation at 5000*g* for 30 min at 4 °C, and the pellet was stored at −80 °C.

2.2. Recombinant AtLASPO purification

The protein was purified by glutathione-based affinity chromatography. After sonication in lysis buffer (50 mM potassium phosphate, pH 8.0, 50 µM FAD, 0.1 mM PMSF and 1 x protease inhibitor cocktail Complete Mini, Roche Diagnostics, Mannheim, Germany), the homogenate was centrifuged at 30000*g* for 30 min at 4 °C. The supernatant was combined with 1 mL pre-washed glutathione resin (GenScript, Nanjing, China) for 2 h at 4 °C. The column filled with resin was washed with 20 mL washing buffer (50 mM potassium phosphate, pH 8.0) and then eluted with fresh 10 mM glutathione elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione). Free glutathione was