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Research article

Biochemical characterization and homology modeling of polyamine oxidase from cyanobacterium *Synechocystis* sp. PCC 6803

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ABSTRACT

The intracellular polyamine contents are regulated not only by polyamine biosynthesis and transport but also by polyamine degradation catalyzed by copper-dependent amine oxidase (DAO) and FAD-dependent polyamine oxidase (PAO). The genome sequence of *Synechocystis* sp. PCC 6803 reveals the presence of at least one putative polyamine oxidase gene, *slr5093*. The open reading frame of *slr5093* encoding *Synechocystis* polyamine oxidase (SynPAO, E.C. 1.5.3.17) was expressed in *Escherichia coli*. The purified recombinant enzyme had the characteristic absorption spectrum of a flavoprotein with absorbance peaks at 380 and 450 nm. The optimum pH and temperature for the oxidation of both spermidine and spermine are 8.5 and 30 °C, respectively. The enzyme catalyzed the conversion of spermine and spermidine to spermidine and putrescine, respectively, with higher catalytic efficiency when spermine served as substrate. These results suggest that SynPAO is a polyamine oxidase involved in a polyamine back-conversion pathway. Based on the structural analysis, Gln94, Tyr403 and Thr440 in SynPAO are predicted to be important residues in the active site.

1. Introduction

Polyamines are small aliphatic polycationic molecules found in both prokaryotic and eukaryotic cells. The most common types of polyamines found in many living cells, including cyanobacteria, are diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm) (Jantaro et al., 2003). They are involved in cell growth, development and adaption against environmental stress (Bouchereau et al., 1999; Bagni and Tassoni, 2001). Intracellular polyamine contents are regulated by not only biosynthesis and transport but also catabolism (Wallace et al., 2003). FAD-dependent polyamine oxidases (PAOs) are involved in polyamine catabolism. They catalyze the oxidation of Spd and Spm and/or their acetylated derivatives at the secondary amino groups generating different reaction products depending on the mode of substrate oxidation. In particular, there are two types of PAOs in plants (Kusano et al., 2015). The plant PAOs of the first type, which are involved in the terminal catabolic pathway, oxidize the carbon on the *endo*-side of the N^5-nitrogen of Spd and Spm to produce 4-aminobutanal and N-(3-aminopropyl)-4-aminobutanal, respectively, together with 1,3-diaminopropane and H_2O_2 (Cona et al., 2006). A *Zea mays* PAO (ZmPAO) (Tavladoraki et al., 1998), an *Oryza sativa* PAO (OsPAO7) (Liu et al., 2014b) and two *Hordeum vulgare* PAOs (HvPAO1 and HvPAO2) (Cervelli et al., 2006) belong to this type of PAOs. The three-dimensional (3D) structure for ZmPAO in the native (oxidized) state was resolved for the first time in 1999 (Binda et al., 1999), and more recently Fiorillo et al. (2011) reported the ZmPAO structures in complex with Spd and Spm. The second type of plant PAOs oxidizes the carbon on the *exo*-side of the N^5-nitrogen of Spm to Spd and/or Spd to Put along with H_2O_2 production and is thus involved in polyamine back-conversion (Tavladoraki et al., 2011). Of these, *Arabidopsis thaliana* PAOs (AtPAO1-5) (Tavladoraki et al., 2006; Moschou et al., 2008; Fincato et al., 2011; Ahou et al., 2014; Kim et al., 2014) and *Oryza sativa* PAOs (OsPAO1 and OsPAO3-5) (Ono et al., 2012; Liu et al., 2014a) have been characterized but there is no structural information available for plant PAOs that catalyze the *exo*-mode of polyamine oxidation. Additionally to the plant PAOs, animal, and yeast PAOs also oxidize

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both acetylated and non-acetylated polyamines via *exo*-mode of polyamine oxidation (Fincato et al., 2011; Cervelli et al., 2015) and the 3D structure of yeast Spm oxidase, Fms1, in complex with Spm provides structural details for the *exo*-mode oxidation (Huang et al., 2005). Although there have been reports on PAOs in several organisms, to our knowledge cyanobacterial PAO has not been reported.

Previously, the biosynthesis and transport of polyamine in unicellular cyanobacterium *Synechocystis* sp. PCC 6803, hereafter *Synechocystis*, have been reported (Jantaro et al., 2003; Incharoensakdi et al., 2010). To advance the knowledge on the metabolism of polyamine in this organism, we focused on polyamine degradation. The genome sequence of *Synechocystis* (Cyanobase; *Synechocystis*) reveals the existence of at least one uncharacterized amine oxidase gene, *slr5093*. In this study, the *slr5093* gene was cloned, expressed in *Escherichia coli* and the obtained recombinant SynPAO was characterized for its catalytic properties and reaction products. In addition, we created a 3D homology model for SynPAO and docked Spm into the catalytic site to find out residues involved in substrate binding. Thereafter, we compared the predicted Spm binding modes of SynPAO and *Mus musculus* spermine oxidase (MmSMO; Tavladoraki et al., 2011) with the known Spm binding modes of yeast Fms1 (*exo*-mode oxidation) and ZmPAO (*endo*-mode oxidation) (Huang et al., 2005; Fiorillo et al., 2011). By combining the results of the phylogenetic and structural analysis of PAOs in both families, we were able to suggest the key amino acids responsible for the substrate binding and *exo*-mode oxidation of SynPAO.

2. Materials and methods

2.1. Preparation of recombinant SynPAO protein in E. coli

The *slr5093* gene encoding SynPAO was amplified from *Synechocystis* genomic DNA by PCR. The specific forward primer (5′-ggaattccatatgatcaggcgacgaag-3′) and reverse primer (5′-ccggtcgacaagattattgatctctttcagc -3′) containing restriction sites of *Nde*I and *Sal*I, respectively were used. The PCR products were cloned into pET28a (+), resulting in a pPAO28a. The recombinant plasmid was sequenced and transformed into *E. coli* BL21 (DE3). To produce SynPAO, the *E. coli* was cultured in Auto-Induction Media Terrific Broth (AIM-TB, Suffian et al., 2017) at 37 °C with shaking at 250 rpm. The culture with OD_{600} = 0.4–0.6 was further incubated at 16 °C for 16 h before harvesting the cell by centrifugation. Cell pellets were resuspended with lysis buffer (50 mM Tris-HCl buffer, pH 8 containing 300 mM NaCl, 20 mM imidazole, and 1 mM PMSF). The cells were disrupted by sonication followed by centrifugation at 12,000 g for 45 min at 4 °C. The crude enzyme in the supernatant was purified using Histrap™ FF column (GE healthcare) according to the manufacturer's protocol and dialyzed against 50 mM Tris-HCl buffer, pH 8. The molecular weight of purified protein was analyzed by SDS-PAGE.

2.2. PAO activity assay and determination of kinetic parameters

The catalytic activity of the SynPAO was determined spectrophotometrically (Tavladoraki et al., 2006). In general, the reaction was started when 2 μg of the purified protein was added to reaction mixture containing 100 mM Tris-HCl pH 8.5, 0.5 mM polyamine substrate, 100 μM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzesulfonic acid and 10 U/ml horseradish peroxidase type II in the total reaction volume of 0.25 ml. The increase of a pink adduct was determined by measuring the absorbance at 515 nm using multiplate reader spectrophotometer. The optimum pH was determined using various buffers; 100 mM acetate buffer (pH range 5–6), 100 mM phosphate buffer (pH range 6–7.5), 100 mM Tris-HCl buffer (pH range 8–9), and 100 mM glycine-NaOH buffer (pH range 9.5–10) in the typical reaction mixture. The optimum temperature and amine oxidase activities toward Put, cadaverine (Cad), Spd, Spm, and N^1-acetyl Spm were determined. The kinetics of PAO activity from SynPAO were investigated as described but using various concentrations of Spd (0–2 mM) and Spm (0–1 mM) in 100 mM Tris-HCl buffer pH 8.5 at 30 °C.

2.3. Analysis of polyamine oxidation products by HPLC

The reaction mixture contained 1 mM Spd or Spm in 100 mM Tris-HCl buffer pH 8.5 at 30 °C. The purified recombinant protein was added to start the reaction then reaction was stopped by adding 5% perchloric acid. The mixture was then derivatized by Benzoylation method (Flores and Galston, 1982). The content of polyamine derivatives were determined by high performance liquid chromatography with inertsil®ODS-3 C-18 reverse phase column (5 μm; 4.6 × 150 mm) using UV–vis detector (254 nm). The mobile phase was run at 0.8 ml/min for 50 min using methanol/water gradient of 50–100% (v/v).

2.4. Sequence alignment for homology modeling and phylogenetic analysis

To find a structural template for modeling the 3D structure of SynPAO, a NCBI BLAST (Basic Local Alignment Search Tool) search (Altschul et al., 1990) was performed using the SynPAO sequence (Slr5093; UniProt code Q6ZEN7) as a query against Protein Data Bank (PDB). The search revealed the structures of ZmPAO (PDB ID: 1B37; Binda et al., 1999) and the amine oxidase domain of human lysine-specific histone demethylase (hLSD2-AOD, residues Lys383-Phe822 of PDB ID: 4FWE; Zhang et al., 2013) as the best templates (with expectation values (e-value) of 3×10^{-51} and 2×10^{-55}, respectively).

Since the sequence identities between SynPAO and the identified template structures were modest in the BLAST results (30% to ZmPAO and 32% to hLSD2-AOD), we first generated a structure-based sequence alignment by superimposing the crystal structures of hLSD2-AOD and of ZmPAO using the program VERTAA (Johnson and Lehtonen, 2000) in Bodil (Lehtonen et al., 2004) to increase the reliability of the residue-residue correspondences in the alignment. Thereafter, we performed the BLAST searches with ZmPAO (UniProt code O64411), hLSD2-AOD (UniProt code Q8NB78), and SynPAO sequences as queries against non-redundant protein sequence database and collected a total of 74 plant, bacterial and mammalian sequences with a sequence identity of 18.3–51.4% to SynPAO (Supplementary Table 1). Of these sequences, we then selected *H. vulgare* PAOs (HvPAO1 and HvPAO2), the amine oxidase domains of lysine-specific histone demethylases from *O. sativa* (OsLSD1) and *A. thaliana* AtLSD1 and AtLSD2 as well as the sequences of *A. thaliana* PAO1 (AtPAO1), hypothetical proteins from *Thiothrix disciformis* (TdPAO) and *Microcystis aeruginosa* (MaPAO) to be aligned together with the SynPAO sequence to the pre-aligned structure-based alignment of the hLSD2-AOD and ZmPAO sequences using MALIGN in the Bodil (Lehtonen et al., 2004). Thus, the generated multiple sequence alignment still contains the structural residue-residue correspondence of ZmPAO and hLSD2-AOD and, with an equal number of sequences similar to ZmPAO, hLSD2-AOD and SynPAO, the produced multiple sequence alignment is much more reliable than any pairwise alignment (Pearson, 2013). Thereafter, the alignment was manually slightly refined to avoid insertions and deletions within the predicted secondary structure elements using the secondary structure