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Structural characterization of a putative recombinant L-amino acid oxidase from *Leptospira interrogans*

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Amino acid oxidases (AOs) are flavin adenine dinucleotide (FAD)-dependent dimeric enzymes that stereo specifically catalyse the deamination of an α -amino acid leading to an α -keto acid. Putative *Leptospira interrogans* recombinant L-amino acid oxidase (*Li-rLAO*; lacking 20 residues corresponding to the N-terminal signal sequence) was cloned, expressed, purified, and its three-dimensional structure was determined by X-ray crystallography at a resolution of 1.8 Å. The active site could be easily identified by the presence of electron density corresponding to a non-covalently bound FAD in both protomers of the dimeric enzyme. Structural analysis of *Li-rLAO* revealed that its polypeptide fold is similar to those of the previously determined homologous structures as available in the Protein Data Bank. However, a substrate-binding residue found at the active site of other previously determined homologous structures was not conserved in *Li-rLAO*, suggesting that its specificity may differ from those of earlier reported structures. Not surprisingly, *Li-rLAO* showed no activity for most amino acids and amines; it exhibited a low activity only with L-arginine as the substrate. The catalytic properties of *Li-rLAO* could be rationalized in terms of its three-dimensional structure.

Keywords: Amino acid oxidase, dimeric enzyme, homologous structures, *Leptospira interrogans*, structural analysis.

LEPTOSPIRES are obligate, aerobic, motile, tightly coiled, spiral-shaped bacteria measuring 0.1 µm in diameter and 6–20 µm in length^{1–6}. Weil observed a condition leading to severe jaundice in people dwelling in marshy areas. In 1914, Inada and Ido identified and confirmed that *Leptospira* was the causative agent leading to severe jaundice (leptospirosis)¹. Kidney is the main target of *Leptospira* sp. Ultrastructural study of mice kidney after infecting with *Leptospira* sp. demonstrated that the species enters by

penetration of the capillary lumen, followed by entry into the interstitial tissue causing oedema and cellular infiltration at days 4–8. *Leptospira* sp. is found in the proximal tubular cells on day 10 of infection^{7–9}. Acute kidney injury resulting from tubulointerstitial nephritis is an early and primary manifestation of systemic leptospirosis¹⁰. The proximal tubule of the kidney is a rich source of amino acids. In most mammals, ~99% of filtered amino acids are reabsorbed in the proximal tubule, which allows utilization of ~70 g amino acids/day in a 70 kg person. The excretion of most amino acids is limited to between 0.2% and 2.5%, although this may increase in various pathologic conditions^{11–17}. *Leptospira* finds a haven here, evading the immune system and utilizing the rich nitrogenous source for survival. Therefore, enzymes that help utilize amino acids in *Leptospira* sp. are likely to be suitable targets for the design of drugs against the pathogen.

L-amino acid oxidase (LAO; EC 1.4.3.2) activity was studied for the first time by Zeller¹⁸ in 1944. In the human kidney, there is an LAO and a D-amino acid oxidase that stereo specifically catalyses the oxidative deamination of almost all L- and D-amino acids. In the kidney, D-amino acid oxidase activity is higher than that of LAO, which is important for detoxifying D-amino acids derived from bacteria and a few other sources^{19,20}. Phylogenetically, LAO from animals are clustered with gastropod enzymes. Biochemical and structural studies have been conducted on LAOs of snake venom (svLAOs), fishes and mammals^{21,22}. svLAOs have been the subject of many studies, and several reviews have been published dealing with their biochemical properties and their importance in pharmacology^{23–25}. svLAOs are usually homodimeric glycoproteins with subunit molecular weights of around 50–70 kDa and use non-covalently linked FAD (flavin adenine dinucleotide) or FMN (flavin mononucleotide) as the cofactor for catalysis^{24,26}. In contrast to svLAO, very little is known about LAOs from microorganisms. In recent years, many bacterial enzymes have been shown to exhibit LAO activity^{20,25}. The bacterial

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enzymes do not cluster into well-defined groups²⁵. The enzymatic properties of LAOs from microbial sources are usually distinct in terms of the optimum pH for catalysis, substrate specificity and stability^{20,27–29}.

The availability of the complete genome sequence for both pathogenic and non-pathogenic *Leptospira* provides an opportunity to explore this organism's metabolic enzymes systematically. Proteomic analysis has provided evidence for a secreted putative L-amino acid oxidase (NCBI ref. sequence: WP_000778112.1 & NZ_LMAQ010000327.1; Uniprot ID: IQ65_19870; UniProtKB entry: A0A098MN43) in cultures of *Leptospira*³⁰. There is no report in the literature on the biochemical characterization of this putative LAO. This 447-residue polypeptide sequence contains an N-terminal putative signal peptide referred to as the TAT (twin-arginine translocation) signal sequence³¹, which may be cleaved after membrane transport. The present study reports the structural and biophysical characterization of the putative *Leptospira interrogans* recombinant L-amino acid oxidase (*Li*-rLAO), lacking 20 residues corresponding to the N-terminal signal sequence.

Materials

Escherichia coli strains Top10, DH5 α , BL21 (DE3) and C41 were a generous gift from A. Pennmatsa (MBU, IISc), pET21b plasmid was a generous gift from M. Vijayan (MBU, IISc). The 6 \times DNA loading dye, dNTPs, protein markers and DNA ladder were procured from Bangalore Genei, India. PHUSION DNA polymerase, pre-stained marker (Thermo Scientific, USA), *Nde*I, *Xho*I, *Dpn*I endonucleases were from NEB. Plasmid isolation kit (Qiagen, Germany), Tris, BSA, FAD, dialysis membrane, ethidium bromide, DTT, agarose, sodium dodecyl sulphate and PMSF were purchased from Sigma, USA. Primers were procured from Eurofins, India; sodium chloride, EDTA, glycerol, acrylamide, bis-acrylamide, bromophenol blue, ammonium persulphate, TEMED, glycine were from SDFCL; amino acid substrates L-histidine, L-alanine, L-tryptophan, L-methionine L-glutamine, L-asparagine, L-glutamate, L-aspartate, L-serine, L-arginine, L-proline and L-tyrosine were from Sigma (a generous gift from P. Balaram (MBU, IISc) and J. Chatterjee (MBU, IISc)); L-phenylalanine, L-leucine, L-isoleucine, L-valine and L-threonine were obtained from Sisco Research Laboratories Pvt Ltd. Amine substrates Dopamine and Spermine (Sigma, USA) were also gifted by A. Pennmatsa (MBU, IISc). Spermidine (Sigma, USA) was a gift from B. Gopal (MBU, IISc); Histamine HCl was from HiMedia, Putrescine, and Tryptamine (Sigma, USA) from P. Balaram.

Methods

Sequence analysis of putative leptospiral LAO

BLASTP and PSI blast³² were used to find sequences in the SWISS-PROT database homologous to *Li*-rLAO. The

motifs present in the sequence of *Li*-rLAO were located using the motif search protocol in Pfam³³ and conserved domain database³⁴. Multiple sequence alignment with homologs was achieved using the UniProt Knowledge database (UniProt, 2017) and UniProt Align³⁵.

Cloning

The polynucleotide corresponding to *Li*-rLAO (without the N-terminal 20-amino acid signal peptide) obtained from the pET21b plasmid containing the full-length *Li*-LAO gene cloned between *Nde*I and *Xho*I sites was amplified by site-directed mutagenesis using Phusion polymerase with the forward primer: ATGGGCCTTCCAGGAATAAAATTAAGTG and the reverse primer: ATGTATATCTCCTTCTTAAAGTTAAAC, under the following conditions: initial denaturation at 95°C for 30 sec, followed by 16 cycles consisting of the following steps: denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, PCR amplification at 68°C for 7 min followed by a final extension step at 68°C for 5 min. The amplified PCR product was treated with *Dpn*I for one hour and directly transformed into DH5 α cells. The clone was confirmed by DNA sequencing.

The pET21b plasmid containing the *Li*-rLAO gene was transformed into *E. coli* strain C41 and grown in Luria Bertani broth (LB) for 8–10 h with 100 μ g/ml ampicillin. The culture was used as a 1% pre-inoculum in LB culture grown at 37°C. After induction with 500 μ M IPTG (when OD_{600 nm} was ~0.6–0.8), the culture was allowed to grow for another 16 h at 30°C. Cells were harvested by centrifugation (20 min, 5440 g, 4°C), resuspended in lysis buffer containing 20 mM Tris-HCl (pH 6.8), 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and 10% glycerol (lysis buffer to bacterial culture volume was in the ratio 1 : 20) and disrupted by sonication (35% amplitude, 20 sec off and 10 sec on, 25 cycles). After centrifugation (45 min, 22,900 g, 4°C) and removal of cell debris, the supernatant was subjected to SP sepharose cation exchange chromatography (0.5 ml/min). The column was pre-equilibrated with 20 mM Tris-HCl (pH 6.8) with a flow rate of 1 ml/min and washed with 20 mM Tris-HCl (pH 6.8). The bound protein was eluted with a linear gradient containing 100 ml of buffer A (20 mM Tris-HCl pH 6.8) and 100 ml of buffer B (20 mM Tris-HCl pH 6.8 with 1 M sodium chloride) with a flow rate of 1 ml/min. Fractions of 10 ml were collected using a fraction collector.

The pooled fractions were concentrated using a 30 kDa cut-off Amicon® Ultra-15 Centrifugal Filter Unit (1060 g, 4°C). The concentrated protein was subjected to gel filtration chromatography (HiLoad 16/600 Superdex 200 pg column, Amersham Biosciences, USA), pre-equilibrated with 20 mM Tris-HCl (pH 6.8) containing 100 mM sodium chloride (ÄKTA Basic 10 HPLC system, GE Healthcare). The purity of the protein obtained by gel filtration was checked on SDS-PAGE. LC-ESI/MS was used to determine the accurate mass of the protein. The concentration of purified protein was estimated by the Bradford method.

Mass spectrometry

Electrospray ionization mass spectra were recorded on maXis impact Q-TOF (Bruker Daltonics, Bremen, Germany) coupled to Agilent 1200 series online HPLC. The spectrometer was tuned using a standard Agilent ESI Tune mix ranging from 118 to 2721 m/z in the positive-ion mode. Data processing was done using the deconvolution module of the Data Analysis 4.1 software (Bruker Daltonics, Bremen, Germany) to identify multiple charge states of the protein and obtain the derived mass.

In-gel trypsin digestion and MS/MS

The bands corresponding to Li-rLAO protein were cut out from Coomassie brilliant blue-stained 10% SDS-PAGE (reducing) gel and subjected to tryptic digestion using sequencing grade modified trypsin (Promega Corporation)³⁶. In brief, stained gel pieces were excised, minced into 1 mm³ size, and transferred to a sterile centrifuge tube. The gel pieces were washed with 500 μ l of wash buffer (50% acetonitrile, 50 mM ammonium bicarbonate) till the Coomassie dye was removed. Destained gel pieces were dehydrated in 100% acetonitrile for 5 min and rehydrated in 150 μ l reduction solution (10 mM DTT, 100 mM ammonium bicarbonate) for 30 min at 56°C. The sample obtained was incubated with 100 μ l of alkylating solution (50 mM iodoacetamide, 100 mM ammonium bicarbonate) for 30 min in the dark at room temperature. Reduced and alkylated gel pieces were washed with wash solution, dehydrated with 100% acetonitrile for 5 min and completely dried at room temperature in a centrifugal evaporator. Gel pieces were rehydrated with 20 μ l of sequencing-grade trypsin (20 μ g/ml) and incubated overnight at 37°C. The digested peptides were extracted from the gel pieces with an extraction solution (50% acetonitrile, 0.1% TFA). The extracted peptides from each sample were further concentrated in a vacuum concentrator and subjected to MS/MS analysis.

Analytical gel filtration

Analytical gel filtration chromatography was performed on a Superdex 200 increase 10/300 GL (GE Healthcare) column to determine the oligomeric state of the purified protein. Gel filtration was done on a pre-equilibrated column at a flow rate of 0.2 ml/min, using 20 mM Tris-HCl buffer (pH 6.8) containing 100 mM NaCl. The protein solution was centrifuged at 22,900 g for 20 min at 4°C, and the clear supernatant was injected into the column. The injection volume was 500 μ l with a protein concentration of ~140 μ M. Absorbances at 220 and 280 nm were recorded to monitor the elution of the protein. Molecular weight standards, carbonic anhydrase (19 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), apo-ferritin (443 kDa), beta-amylase (200 kDa) and thyroglobulin (669 kDa) were

used to calibrate the column pre-equilibrated with 50 mM tris-HCl buffer (pH 7.2) containing 100 mM KCl with a flow rate of 0.2 ml/min.

Circular dichroism spectrum and thermal denaturation

The circular dichroism (CD) spectrum was recorded on a JASCO-715 spectropolarimeter (JASCO technologies, Tokyo, Japan) equipped with a thermostat cell-holder controlled by a Peltier device using a 2 nm bandpass and a cuvette of 1 mm path length. The purified protein (~90 μ M in 20 mM tris-HCl, pH 6.8) was maintained at different temperatures (25°–65°C at 5°C temperature intervals) for thermal melting studies. Three CD scans were recorded at a scan speed of 10 nm min⁻¹ at each temperature. The HT voltage remained in the permissible range throughout the scans.

Spectroscopic characterization

The fluorescence spectra of the purified protein were recorded on an F2500 fluorescence spectrophotometer (Hitachi Science and Technology, Japan) using a protein concentration of ~285 μ M, with excitation wavelengths of 450 and 280 nm. Spectra were recorded in the range 480–800 nm and 300–800 nm respectively.

Crystallization

The purified protein was concentrated to ~20 mg/ml, centrifuged at 22,900 g for 1 h at 4°C and screened for crystallization conditions using Hampton crystal screen kits by the under-oil method. Crystals were obtained in the 95th condition of the index screen in a crystallization drop containing 1 μ l of (~20 mg/ml) protein and 1 μ l of crystallization condition (HR2-944-95 containing 0.1 M potassium thiocyanate, 30% w/v polyethylene glycol monomethyl ether 2000) at 298 K. Initially the crystals were small in size due to excessive nucleation. To overcome this, the condition was diluted with 1 μ l of water before mixing with the protein solution. Good quality crystals appeared in about two weeks.

Data collection, structure solution and refinement

A native crystal was mounted on a cryo-loop and flash-frozen in liquid nitrogen. X-ray diffraction data were collected on the Beamline XRD1/XRD2 at Elettra Synchrotron Trieste, Italy, with the oscillation angle set to 0.5°. The native crystal diffracted to 1.78 Å resolution. Data were processed with iMOSFLM and XIA2-DIALS of the CCP4i2 suite of programs^{37–42}. The intensity data obtained were used for structure determination by molecular replacement using Phaser, MrBUMP, MolREP and the automated

Table 1. Diffraction data and refinement statistics of putative *Leptospira interrogans* recombinant L-amino acid oxidase (Li-rLAO)

Parameters	Li-rLAO (KI-soaked)	Li-rLAO (native)
Wavelength (Å)	1.5498	1.07
Resolution limits (Å)	69.68–1.95 (1.95–1.95)	84.61–1.78 (1.81–1.78)
Unit cell dimensions (Å)	51.776, 105.915, 139.363 ($\alpha = \beta = \gamma = 90$)	51.75, 106.22, 139.91 ($\alpha = \beta = \gamma = 90$)
Space group	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁
Rmerge (%)	14.1 (15.9)	11.8 (39.0)
No. of unique reflections	44,824	74,369
No. of molecules in the asymmetric unit	2	2
Completeness (%)	78.6 (11.7)	99.1 (99.8)
Multiplicity	22.1 (3.2)	11.3 (12.1)
Mean ($\langle I \rangle > \alpha(I)$)	16.2 (0.6)	14.3 (5.9)
Rwork/Rfree (%)	–	17.7/22.3
RMSD bond length (Å)	–	0.0147
RMSD bond angle (°)	–	1.834
Average B-factors (Å ²)		
Protein	–	17.18
Water	–	25.08
Ligands	–	9.14
Ramachandran statistics (%)		
Favoured region	–	94.2
Allowed region	–	5.55
Outliers	–	0.25

The values in parenthesis correspond to the highest resolution range.

structure determination server AUTORICKSHAW^{43,44}, utilizing svLAO(PDB id: 2IID, 3KVE, 1REO) as the phasing model. However, none of the molecular replacement programs provided an acceptable solution. Further, the data were also examined in MarathonMR, which uses various domains available in the domain database as models for structure determination⁴⁵. Even these efforts were unsuccessful.

In order to obtain a solution for the crystal structure using a possible signal from a suitable anomalous scatterer, crystals were soaked in a crystallization cocktail containing 0.5 M potassium iodide (1 M of potassium iodide mixed with the crystallization condition in a 1 : 1 ratio) for 15 min, mounted on a cryo-loop, flash-frozen in liquid nitrogen and X-ray diffraction data were collected on the Beamline XRD1/XRD2 at Elettra Synchrotron, Trieste. Data were collected twice for the same crystal with 0.5° oscillation; both the data were merged after renaming the files. The merged data were processed with iMOSFLM and XIA2-DIALS of the CCP4i2 suite of programs^{37–42}. The crystal soaked with KI diffracted to 2.38 Å resolution. Iodine sites in the crystal were located using the anomalous signal, and the substructure of iodine atoms was determined by Crank 2.0 of the CCP4i2 package^{46–53}. The program SHELX identified nine iodine atoms. An initial model with 829 residues ($R = 0.27$; $R_{\text{free}} = 0.35$) was built using Crank 2.0. Manual model building and refinement of the structure were carried out using Coot⁵⁴ v0.7.2.1 and REFMAC (version 5.5.0109)^{55–60} respectively. The refined model was used as a template for molecular replacement with a native

data set^{61,62}, which had diffracted up to 1.78 Å. Further, manual model building and refinement were carried out to obtain the final model with an R -factor of 0.18 and R_{free} of 0.22. The asymmetric unit was compatible with two protomers and 39% solvent content. The data between 84.64 and 1.78 Å were included throughout the refinement calculations. Five per cent of the data was randomly chosen for free R -factor calculation. Table 1 shows the data collection, processing statistics and refinement parameters. The Li-rLAO coordinate file has been deposited in the Protein Data Bank (PDB ID: 7EME).

Structural analysis

Solvent-accessible area and interface residue analysis, accessible and buried surface areas and ΔG for dimer dissociation were calculated using the PISA server^{63–65}. Structures similar to Li-rLAO were identified using DALI^{66–69}, and the top hits were quantitatively compared to the structure of Li-rLAO using the SSM superpose option of Coot⁵⁴. Ramachandran map for the structures was generated using Procheck and Rampage. All molecular illustrations were prepared using Pymol, version 1.2r1.

Amino acid oxidase assay

The assay was conducted in a 96-well microplate in duplicates, following the procedure described by Kishimoto and

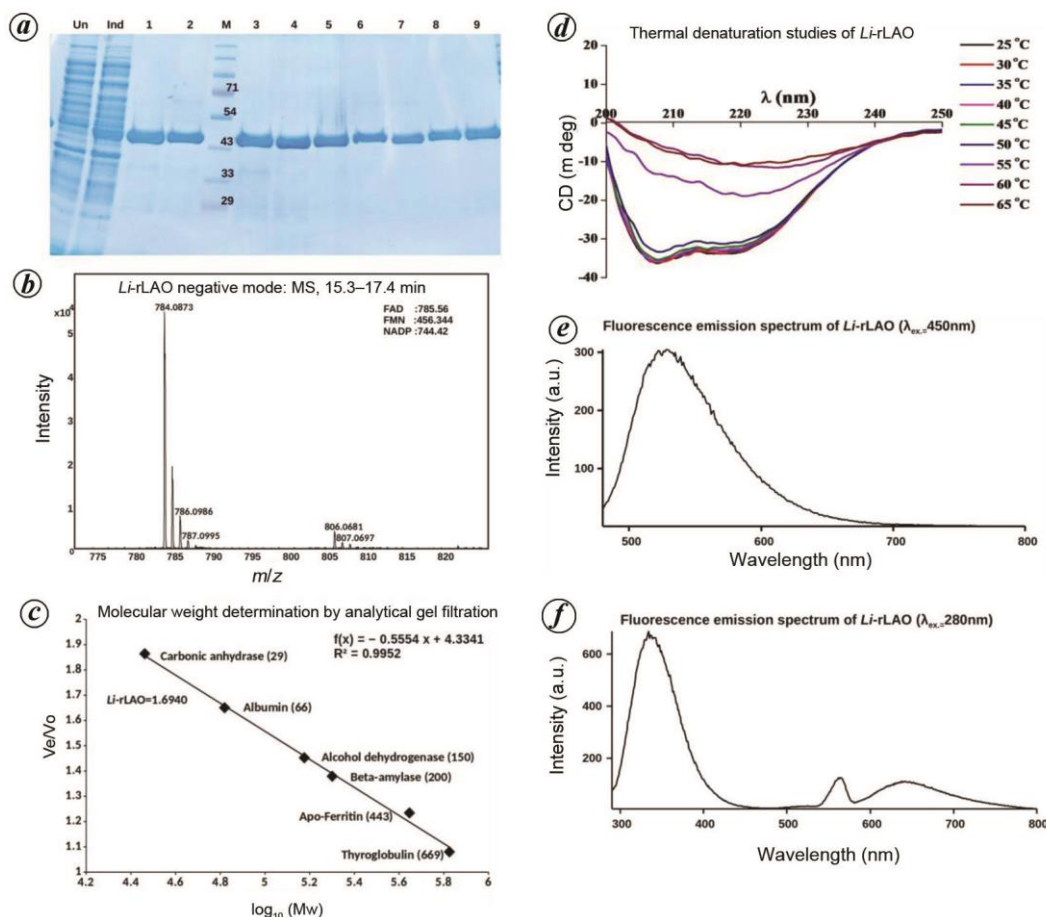


Figure 1. Purification and biophysical characterization of *Leptospira interrogans* recombinant L-amino acid oxidase (*Li-rLAO*). **a**, Representative SDS-PAGE showing expression and purification of *Li-rLAO*. Lanes Un, Ind and M represent uninduced, induced and protein molecular weight markers respectively. The purified *Li-rLAO* fractions after gel filtration (HiLoad 16/600 Superdex 200 pg) chromatography are labelled 1–9. **b**, Mass of flavin adenine dinucleotide (FAD) observed in purified *Li-rLAO* protein in the negative mode. **c**, Standard graph obtained from analytical gel filtration column (Superdex 200 increase 10/300 GL) chromatography using molecular weight markers. **d**, CD spectrum and thermal melting as monitored by recording CD spectra of *Li-rLAO* at different temperatures. **e**, **f**, Fluorescence emission spectra of *Li-rLAO* when excited at (**e**) 450 nm and (**f**) 280 nm.

Takahashi⁷⁰. The standard reaction mixture contained L-amino acid substrate (initially 5 or 10 mM, at later stages, reduced to 2.5 mM), *Li-rLAO* (various concentrations ranging from 10 to 30 μ M), 2 mM ortho-phenylene diamine (OPD), 0.81 U/ml horseradish peroxidase (HRP) and 50 mM borax–HCl buffer (pH 8.5) in a total volume of 100 μ l/well. After incubation at 30°C for 45 min, the absorbance of the reaction mixture was measured by a microplate reader at 420, 492 and 630 nm. Further, the assay was also carried out in a phased manner, with amino acid substrates incubated with *Li-rLAO* (10–30 μ M) for 45 min at 30°C followed by the addition of HRP and OPD and incubation at 30°C for another 45 min, making measurements after 90 min. The assay was carried out with all natural L-amino acids, except for L-cysteine and L-lysine.

Amine oxidase assay

The assay was conducted in a 96-well microplate in duplicate following the procedure described by Holt and Palcic

with modifications⁷¹. The reaction was carried out in a mixture containing 50 mM HEPES, 5 mM KCl, 2 mM CaCl_2 , 1.4 mM MgCl_2 , 140 mM NaCl pH 7.4, 200 μ M substrate, 500 μ M 4-aminoantipyrene, 1 mM vanillic acid, 4 U ml^{-1} horseradish peroxidase and various concentrations of *Li-rLAO* (10–30 μ M) for 30 min at 37°C. The absorbance of the reaction mixture was measured using a microplate reader at 498 nm. Putrescine, histamine, spermine, spermidine, dopamine and tryptamine were used as substrates in the assay.

Results

Sequence analysis

BLAST results suggested low amino acid sequence identities between *Li-rLAO* and other oxidases from various sources. The sequence alignment of LAOs from various organisms using PSI-BLAST indicated that *Li-LAO* aligned well with FAD-dependent oxidoreductases from bacteria.

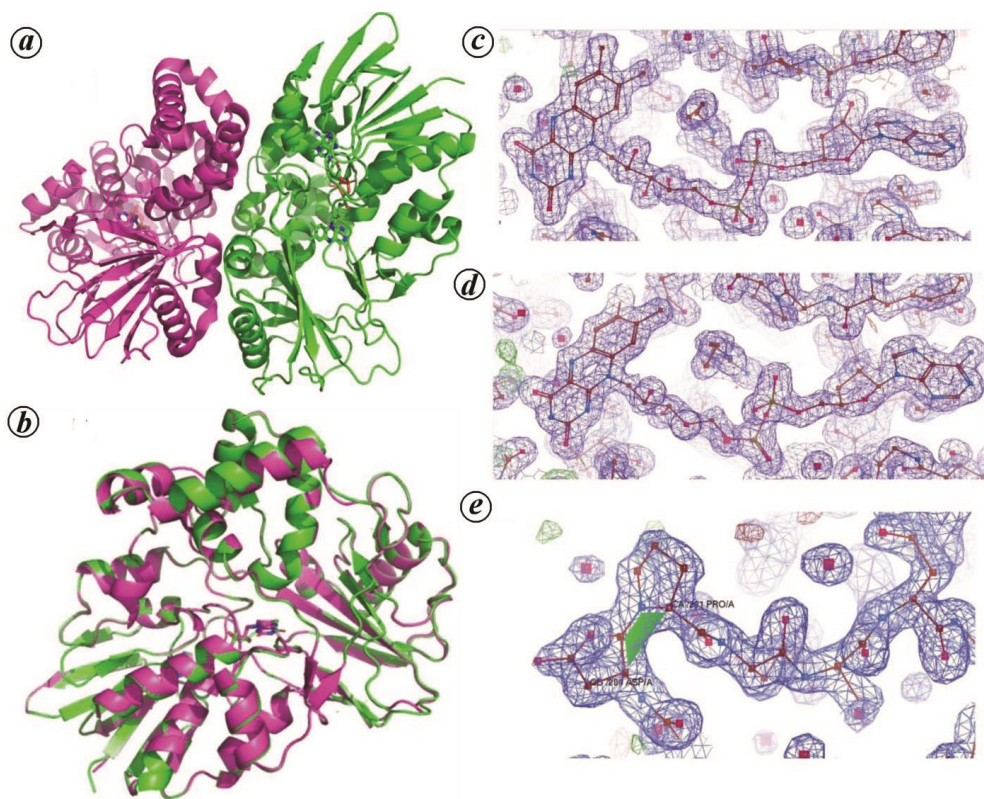


Figure 2. Structure of *Li-rLAO*. *a*, Dimeric structure with subunit A represented in green and subunit B represented in pink colour. *b*, Structural superposition of two subunits *Li-rLAO* illustrating the close similarity of their polypeptide folds. *c*, 2Fo–Fc electron density corresponding to FAD bound to chain A of *Li-rLAO*. *d*, 2Fo–Fc map of FAD bound to chain B of *Li-rLAO*. *e*, Cis-peptide at Asp280 and Pro281 in the polypeptide fold of *Li-rLAO*.

Further, a search against Pfam and Conserved Domain Database (CDD) suggested that *Li-rLAO* may belong to the amino oxidase superfamily (data not shown).

Biochemical characterization

The *Li-rLAO* protein was purified from the lysate of *E. coli* C41 strain by SP-sepharose column followed by S200-Gel filtration chromatography. The yellow-coloured protein was easily collected from the protein-bound SP-sepharose column. Protein purification was monitored by 280 nm absorption of fractions obtained by gel filtration chromatography. The fractions with significant absorption were checked for purity on 10% SDS-PAGE and pooled (Figure 1 *a*). The molecular mass of the purified protein was obtained from Q-TOF mass spectrometry. The protein showed a mass of 47,090 Da, which is 131 Da less than the calculated mass based on sequence, indicating that the N-terminal methionine was probably removed in the *E. coli* strain used (Supplementary Figure 1). Also, unlike mammalian enzymes, the protein is unlikely to be glycosylated. The protein was also subjected to mass spectrometry in the negative-ion mode, and the presence of FAD in the purified protein was confirmed by the molecular weight obtained (Figure 1 *b*). Further, the oligomeric state of the purified protein

was established as a monomer by analytical gel filtration column chromatography (Figure 1 *c*). The identity of the purified protein was further confirmed by tryptic digestion followed by Q-TOF mass spectrometry and data analysis using the Mascot software (data not shown).

The CD spectrum suggested that the protein was well-folded. Thermal melting studies showed that the protein was stable till 50°C. At 55°C, it showed sharp melting (Figure 1 *d*). In the fluorescence spectrum of *Li-rLAO*, the bound FAD showed an absorption band at 450 nm (data not shown) and a broad fluorescence emission band with $\lambda_{\text{max}} = 525$ nm (Figure 1 *e*). The internal tryptophan fluorescence studies of purified protein showed a broad fluorescence emission band with $\lambda_{\text{max}} = 330$ nm when excited at 280 nm and two more peaks at 560 nm (probably corresponding to $280 \times 2 = 560$ nm) and a peak at 640 nm (probably corresponding to a FAD radical; Figure 1 *f*)⁷². Further studies are needed to confirm the identity of these peaks.

Structural features

The X-ray diffraction (XRD) data on a *Li-rLAO* crystal were obtained using beamline XRD1/XRD2 at the Elettra Synchrotron Trieste. Structure determination and refinement followed standard crystallographic protocols (Table 1).

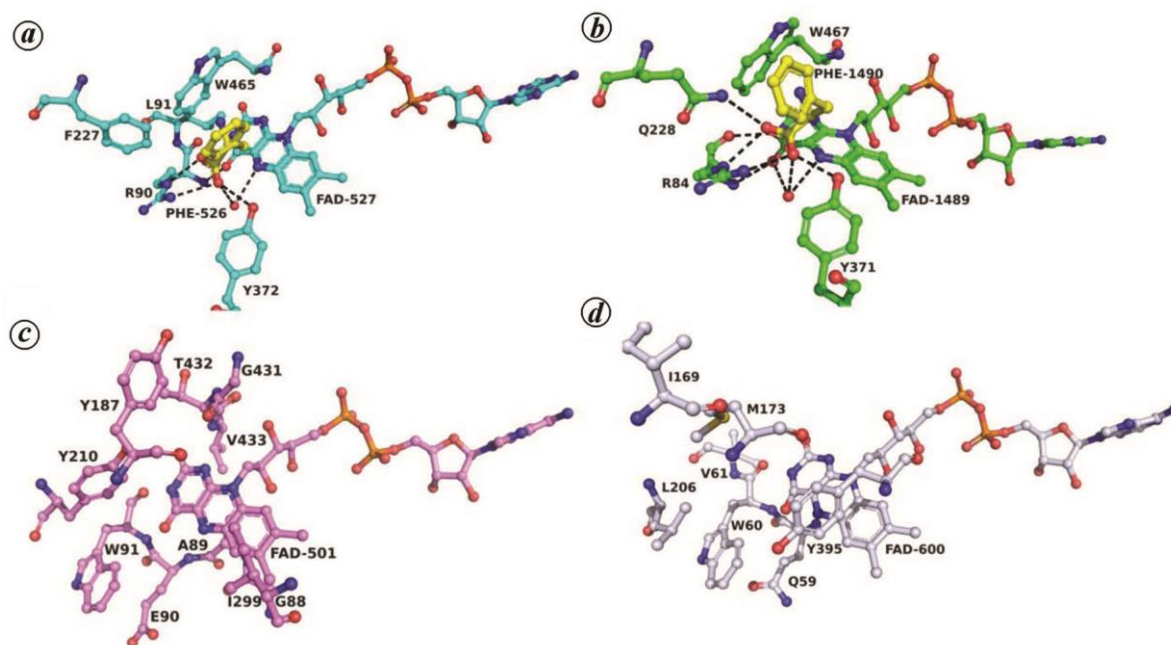


Figure 4. Structural comparison of LAOs. *a* and *b*, Active-site residues with the bound amino acid substrate (yellow) in *Calloselasma rhodostoma* (PDB id: 2IID) and *Rhodococcus opacus* (PDB id: 2JB2) LAO respectively. (Hydrogen bonding between the arginine residue at the active site and the bound amino acid substrate (Phe) could be observed.) *c*, Active site of leptospiral LAO. *d*, Active site of *Rhodococcus erythropolis* putrescine oxidase (PDB id: 2YG3).

($\beta 3$ – $\beta 5$, $\beta 7$ and $\alpha 2$ or black). Structural superposition using Coot⁶⁰ of *Li*-rLAO and other oxidases from various sources revealed the highest sequence identity (24.9%) with LAO from *Rhodococcus opacus* and the lowest (19.6%) with cyclohexylamine oxidase from *Brevibacterium oxydans*. Despite these low sequence identities, the overall three-dimensional structural fold of these proteins was remarkably similar, with the highest similarity from rat monoamine oxidase A (2.14 Å RMSD) and the lowest similarity from *Calloselasma rhodostoma* LAO (2.7 Å RMSD). Thus, these oxidases constitute a set of proteins with low amino acid sequence identity but high structural similarity. This observation is consistent with the diversity of substrates on which these oxidases are functional.

Li-rLAO contains the well-studied FAD-binding fold with a four-stranded, parallel β -sheet ($\beta 1$ – $\beta 2$ – $\beta 8$ – $\beta 12$) sandwiched between a three-stranded, antiparallel β -sheet ($\beta 9$ – $\beta 10$ – $\beta 11$) and an α -helix ($\alpha 1$). Strong hydrogen-bond networks hold FAD. The N1 atom of the adenine moiety of FAD forms a hydrogen bond with the backbone –NH atom of Val240. The N3 atom of adenine moiety of FAD forms a hydrogen bond with the backbone –NH atom of Ala66. The carboxyl group of Glu65 forms hydrogen bonds with –OH groups of ribofuranose ring (Figure 3 *b*). The N6 atom of adenine forms a hydrogen bond with the backbone –C=O of Val240 and sidechain amide (–C=O) of Gln273. The phosphate moiety attached to the ribofuranose forms a hydrogen bond with a conserved Arg73 side chain and its backbone –NH. The phosphate attached to the ribityl moiety forms hydrogen bonds with the back-

bone –NH of Ser46 and its side chain –OH group. This serine is also present in putrescine oxidase, but is substituted by threonine in most flavin-dependent oxidoreductases. Further, a backbone –NH of Glu424 also forms a hydrogen bond with the phosphate moiety. The ribityl moiety forms an internal hydrogen bond with hydroxyl groups at C2' and C4' positions, and with a conserved water molecule surrounding it. The peptide stretch Leu87–Ala89 is at a distance of about 4 Å from the isoalloxazine ring. It may form a barrier to the entry of molecules from the bulk solvent towards FAD. The isoalloxazine ring of FAD is held by hydrogen bonding only with the backbone atoms of the protein chain. The N1 atom of the isoalloxazine ring is not involved in hydrogen bonds. Carbonyl group at C2 forms a hydrogen bond with the backbone –NH of Val433 and two water molecules. One of these water molecules forms a hydrogen bond with His96. The N3 atom forms a hydrogen bond with the backbone –C=O of Trp91. The N5 atom of the isoalloxazine ring forms a hydrogen bond with a water molecule, which in turn is involved in a hydrogen bond network with Lys301 and Glu90. However, these interactions are not conserved in all other FAD-dependent oxidases. The guanidino group of Arg interacting with the phosphate of FAD and the side chain carboxyl group of a Glu interacting with the hydroxyl group of ribofuranose are common features of all structures. Similarly, the adenine moieties N1 and N6 interacting with a Pro and Val are also observed in most of these oxidases. The FAD-interacting residues seem more conserved on the adenine ringside than on the isoalloxazine ringside. The variation

of residues interacting with the isoalloxazine ringside appears to be significant in the evolution of FAD-dependent enzymes and their numerous functions, as these variations facilitate the accommodation of several substrates. These results are consistent with the earlier observation that LAOs do not cluster into well-defined groups²⁵.

Enzymatic activity

Li-rLAO showed no activity with most amino acid substrates, except for a low and reproducible activity with the amino acid substrate L-Arg. It is interesting to note that *Li*-rLAO was active up to 2 mM concentration of L-arginine. Above this concentration, enzyme activity was inhibited (data not shown).

Comparison of the active-site geometries of amino acid oxidases

Due to the absence or low activity of *Li*-rLAO with most of the L-amino acid substrates, the active site of *Li*-rLAO was examined and compared with those of the well-studied *C. rhodostoma* LAA (PDB id: 2IID) and *R. opacus* LAO (PDB id: 2JB2). Figure 4 *a* and *b* shows the residues that interact with FAD and those within 4 Å distance that could interact with the amino acid substrates in *C. rhodostoma* and *R. opacus* LAO respectively. The amino acid sequences of *C. rhodostoma*, *R. opacus* and *Li*-rLAO sequences were aligned, and equivalent residues likely to interact with

substrates were identified (Supplementary Figure 2). In LAOs, a conserved arginine residue forms a hydrogen bond with the carboxyl group of the amino acid substrate. This residue is present in *R. opacus* and *C. rhodostoma* LAOs, but not in *Li*-rLAO. A comparison of the active site geometries showed that the structurally equivalent residue was glutamate in *Li*-rLAO (Figure 4 *c*) and glutamine in putrescine oxidase (Figure 4 *d*). The arginine in *R. opacus* and *C. rhodostoma* LAOs presumably is essential for the activity of amino acid oxidases. Therefore, it is not surprising that *Li*-rLAO is not active towards most amino acid substrates. The *Li*-rLAO active site exhibited greater structural similarity with amine oxidase than amino acid oxidases. Therefore, *Li*-rLAO was tested for activity with amine substrates. However, the enzyme did not show activity with any of the amines examined.

Discussion

Catalysis by LAO requires three substrates – L-amino acid, molecular oxygen and a water molecule. The resulting products are an α -keto acid, hydrogen peroxide and an ammonium ion. The overall reaction of LAO has been described as two half-reactions⁷³. In the first half of the reaction (also called the reductive half), FAD is reduced to FADH₂, leading to the oxidation of the bound amino acid to an imino acid^{74,75}. The second half involves the oxidation of FADH₂, in which an oxygen molecule enters the active site of the enzyme, abstracts the hydrogen atoms from FADH₂ and gets converted to hydrogen peroxide, which exits from the active site. The different ways in which molecules enter and exit the active site during the reaction cycle have been studied. It has been suggested that lining of water molecules at the active site of LAO represent the entry–exit paths. It was possible to identify water-filled channels in *Li*-rLAO using the CAVER 3.03 program⁷⁶. A tunnel in the interface is formed by chain A (residues Leu292–Leu298, Gly347, Asp348 and Pro402–Gly404) and chain B (residues Arg160–Asn165 and Asp190–Arg193; Figure 5 *a*); a second path constituted by peptide stretches Leu429–Leu437, Ile92–Ala99, Asn206–Tyr210, Ser320–Phe326, Asn181–Tyr187 and residues Ile103, Leu108, Lys109, Val214, Glu215, Leu221, Met218, Val299, Arg295, Arg349 and Ile398; another tunnel leading to FAD and limited by the peptide stretches Asn181–Ser191, Phe113–Val115, Ser320–Phe326 and Ile344–Arg349 and residues Tyr210 and Ile299, and a fourth path formed by the peptide stretches Gly347, Asp348, Tyr296–Arg298, Ala399–Phe401, Tyr187–Asp190 and Glu428–Asn430 and residues Leu195, Lys199 and Asp203 (Figure 5 *b*) could be identified. Despite the presence of these channels, *Li*-rLAO is not active with amino acid substrates, suggesting that the changes in residues close to the active site, in particular the absence of arginine conserved in other L-amino acid oxidases, are probably responsible for the lack of activity.

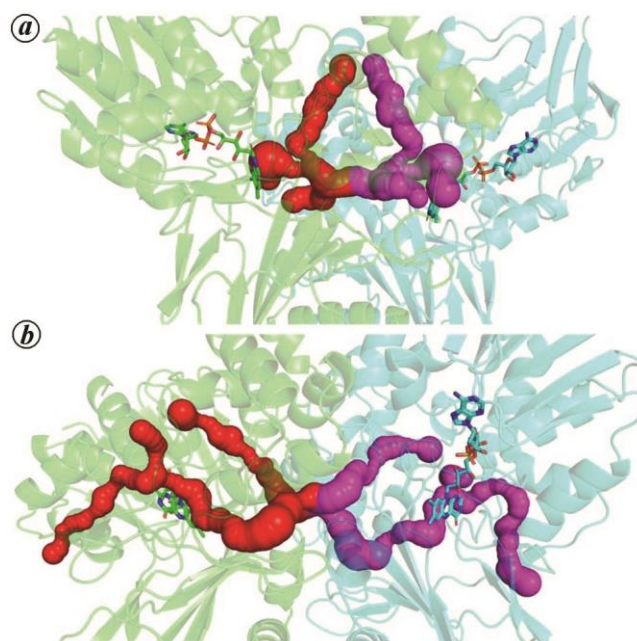


Figure 5. Identification of water-filled channels leading from bulk solvent to the active site of *Li*-rLAO using the CAVER tool. *a*, Tunnels present in the interface of the crystal dimer of *Li*-rLAO. *b*, Tunnels that lead to the active site of *Li*-rLAO.

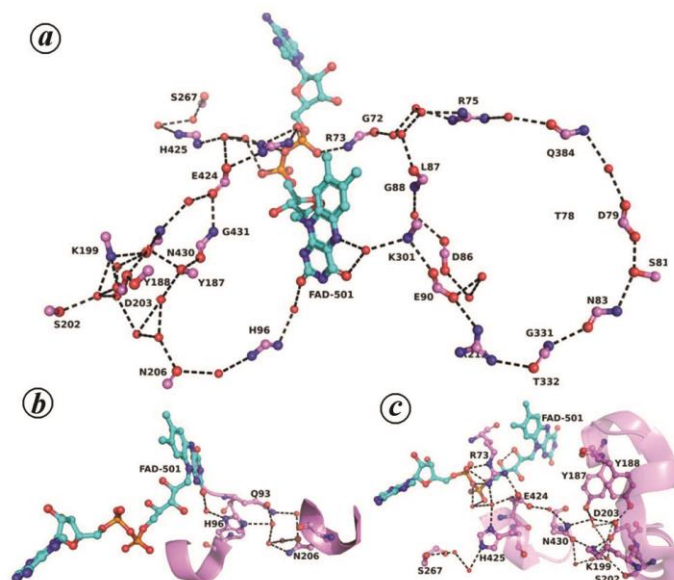


Figure 6. Structural analysis of *Li-rLAO*. *a*, Hydrogen-bond networks observed in the *Li-rLAO* structure. *b*, His95 forms a hydrogen bond network with C2 (C=O) of FAD. *c*, His425 forms a hydrogen-bond network with the phosphate group of FAD.

Although *Li-rLAO* may catalyse a unique reaction distinct from the oxidation of amino acids, similarity in the structure of *Li-rLAO* and other amino acid oxidases suggests that it is worthwhile to consider the functional mechanism of LAOs. As mentioned earlier, in LAO, the reduction of FAD is proposed to follow one of the two mechanisms mentioned below. In the first mechanism, a proton is transferred to FAD from the C α -atom of the substrate, leaving a negative charge on the carbon atom. This is followed by a two-electron transfer⁷⁷. In the second mechanism, the C α -hydrogen atom is transferred as a hydride ion carrying two electrons by a hydride transfer pathway⁷³. In both these mechanisms, one hydrogen atom is added to each of the N1 and N5 atoms of the isoalloxazine ring. It has been proposed that there could be a direct translocation of proton/hydride ions to N5 of the isoalloxazine ring of FAD. With this background, it would be interesting to examine the plausible mechanism of a transfer of a proton/hydrogen atom from the substrate to the N1 atom of the isoalloxazine ring of FAD in LAOs. In *Sv-LAO*, His223 has been suggested to abstract the proton from the amino group of the substrate^{78–80}. In the crystal structure of *C. rhodostoma* LAO, a short pathway that may be involved in the proton translocation has been observed from His223 to N1 of FAD. The residue structurally equivalent to His223 is not conserved in *Li-rLAO*. In spite of this change, two hydrogen-bond networks that link the substrate-binding residues to the oxygen atom of the C2 keto group of the isoalloxazine ring could be identified in *Li-rLAO* (Figure 6 *a*). The exciting aspect of these networks is that the water molecules positioned between polar side chains may facilitate proton translocation. These hydrogen-bond networks do not involve peptide backbone atoms, except for the pep-

tide bond containing Gly residues. The flexibility due to the occurrence of glycine may facilitate the formation of this hydrogen bond network⁸¹. There are only two histidine residues in *Li-rLAO*. His96 forms a hydrogen bond with water, forming a hydrogen bond with the carbonyl of the second carbon atom of the FAD isoalloxazine ring (Figure 6 *b*). His425 forms a hydrogen-bond network with a water molecule, forming a hydrogen bond with the phosphate group of FAD (Figure 6 *c*). The significance of these histidine residues in *Li-LAO* is yet to be established. The absence of residues considered essential for the activity of *Li-rLAO* with amino acid substrates suggests that *Li-rLAO* may act on an unusual substrate. Further studies are needed for the identification of the reaction catalysed by *Li-rLAO*.

Conclusion

The putative *Li-rLAO* was cloned, expressed and purified to homogeneity and characterized by various biophysical methods. The structure of the recombinant protein was obtained by X-ray crystallography to a resolution of 1.8 Å. The polypeptide fold of *Li-rLAO* resembled that of the evolutionarily well-conserved FAD-binding amino acid oxidases. On the contrary, *Li-rLAO* lacked a conserved substrate-binding residue (arginine) compared with known LAO structures. *Li-rLAO* did not exhibit any activity with most of the L-amino acids, but had low activity only with L-arginine. *Li-rLAO* exhibited little or no activity with amines, including putrescine, spermine and spermidine. Further studies are needed to determine the specific biological role of *Li-rLAO*.

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