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Structural insights into selectivity and cofactor binding in snake venom L-amino acid oxidases

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ABSTRACT

L-Amino acid oxidases (LAAOs) are flavoenzymes that catalytically deaminate L-amino acids to corresponding α-keto acids with the concomitant production of ammonia (NH_3) and hydrogen peroxide (H_2O_2). Particularly, snake venom LAAOs have been attracted much attention due to their diverse clinical and biological effects, interfering on human coagulation factors and being cytotoxic against some pathogenic bacteria and *Leishmania* ssp. In this work, a new LAAO from *Bothrops jararacussu* venom (BjsuLAAO) was purified, functionally characterized and its structure determined by X-ray crystallography at 3.1 Å resolution. BjsuLAAO showed high catalytic specificity for aromatic and aliphatic large side-chain amino acids. Comparative structural analysis with prokaryotic LAAOs, which exhibit low specificity, indicates the importance of the active-site volume in modulating enzyme selectivity. Surprisingly, the flavin adenine dinucleotide (FAD) cofactor was found in a different orientation canonically described for both prokaryotic and eukaryotic LAAOs. In this new conformational state, the adenosyl group is flipped towards the 62–71 loop, being stabilized by several hydrogen-bond interactions, which is equally stable to the classical binding mode.

1. Introduction

A number of proteins, enzymes and biologically active peptides that interfere with key physiological processes are present in snake venoms, triggering a wide spectrum of secondary effects such as blood clotting, myotoxicity, neurotoxicity, platelet aggregation and lipid digestion [1,2]. Proteins that act at specific points and interfere with the highly-regulated blood coagulation cascade and platelet aggregation have been recruited to serve as diagnostic and clinical tools [3,4].

L-Amino acid oxidases (LAAOs; E.C. 1.4.3.2) are flavoenzymes that catalytically deaminate L-amino acids to the corresponding α-keto acids with the production of ammonia (NH_3) and hydrogen peroxide (H_2O_2) [5,6]. LAAOs are cytotoxic proteins [7,8], which inhibit platelet aggregation [8,9] and are active against *Leishmania* spp. [10], bacteria, and viral proteins [5–11]. Since the bactericidal activity of LAAO is inhibited by catalase, this suggests that hydrogen peroxide is important in these processes [11].

LAAOs can be inactivated by decreasing the pH and can be reactivated by increasing pH [12] and inactivation by freezing is more pronounced between −20 and −30 °C [13]. These results suggest that inactivation of the enzyme is likely due to conformational changes in the protein structure, particularly around the flavin binding site [13].

Although their content varies between genera and species, most of the Viperidae venoms contain LAAOs. In *Bothrops* species, LAAOs represent approximately 2% of the total weight of the desiccated crude venom and leads to the typical yellow color [10,11]. Although LAAOs have been isolated from different organisms, snake venom LAAOs are the best characterized [7]; however, there is as yet no clear correlation between their structural and toxic properties. Here we present the results of the purification and structural characterization of a new L-amino acid oxidase from the venom of *Bothrops jararacussu* (BjsuLAAO) to improve this correlation.

2. Materials and methods

2.1. Two-step purification procedure

Desiccated crude venom (125 mg) was suspended in 1.5 ml of 0.02 M Tris–HCl buffer containing 0.15 M NaCl, pH 8.0 and centrifuged at 10,000×g for 10 min. The clear supernatant (1 ml) was applied onto a 16/60 Sephacryl S-100 column previously equilibrated with the aforementioned buffer. The protein fractions were eluted

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at a flow rate of 0.2 ml/min and fractions of 1 ml/tube were collected, the absorption was monitored at 280 nm and the fractions were analyzed by SDS–PAGE [14]. The fractions forming the second peak of size-exclusion chromatography (SEC) were pooled and were concentrated to 0.5 ml using a micro-concentrator (AMICON) with a 30 kDa membrane (Fig. S1) and applied onto a Mono Q 5/50 GL column. The column was washed with 0.02 M Tris–HCl buffer pH 8.0 (eluent A) and eluted with a nonlinear salt gradient (eluent A + 1 M NaCl). Protein concentration was determined according to a microbiuret method described by [15], using bovine serum albumin as standard.

2.2. Substrate specificity

Substrate specificity was determined by dissolving 2 mM of the amino acids L-Histidine, L-Glutamine, L-Threonine, L-Serine, L-Lysine, L-Arginine, L-Phenylalanine, L-Tryptophan, L-Leucine, L-Isoleucine, L-Methionine, L-Cystine, L-Cysteine, L-Valine and L-tyrosine in 500 μl of 0.1 M Tris–HCl pH 7.2 with the addition of 5 μl of solution containing *O*-phenylenediamine (OPD) (10 mg/ml) and 1 μl of peroxidase (1 mg/ml). The final solution was incubated with 3 μg of enzyme for 30 min at 25 °C. The reaction was blocked with 500 μl of 10% citric acid (w/v) and the absorbance was determined at 490 nm.

2.3. Crystallization

The purified LAAO from *B. jararacussu* (BjsuLAAO) was concentrated to 9 mg/ml in micro-concentrators (AMICON MWC 30 kDa) and stored in a 0.02 M Tris–HCl pH 8.0 buffer at 4 °C. Crystallization was performed by the hanging-drop vapor-diffusion method using 24-well tissue-culture plates [16] and commercially available crystallization screens such as crystal screen 1 and 2, polyethylene glycol 6000, ammonium sulfate kits (Hampton research) and the PEG suite (Quiagen). Typically, 1 μl of a protein solution was mixed with an equal volume of the screening solution and equilibrated over a reservoir containing 0.5 ml of the latter solution. Crystals suitable for diffraction were obtained with 0.1 M sodium acetate trihydrate pH 4.6 and 25% (w/v) polyethylene glycol 1000.

2.4. Data collection and structure determination

A BjsuLAAO crystal was directly flash-cooled in a 100 K nitrogen-gas stream and X-ray diffraction data were collected on the W01B-MX2 beamline at the Brazilian Synchrotron Light Laboratory (Campinas, Brazil). The wavelength of the radiation source was set to 1.458 Å and a MarMosaic 225 mm CCD detector was used to record the X-ray diffraction intensities. The data were indexed, integrated and scaled using the DENZO and SCALEPACK programs from the HKL-2000 package [17]. Molecular replacement was carried out using the MOLREP program [18] and a model based on the atomic coordinates of native L-amino acid oxidase from *Vipera amodytes amodytes* (PDB code 3KVE) [2].

3. Results and discussion

BjsuLAAO was isolated from the *B. jararacussu* venom by two chromatographic steps (SEC and AEC) yielding 5 mg of purified enzyme from an initial amount of 250 mg of crude venom (Figs. S1–S4).

3.1. BjsuLAAO has higher specificity for hydrophobic residues

The BjsuLAAO activity was tested using different amino acids as substrate (Fig. 1). The enzyme showed high activity for aromatic and aliphatic amino acids with large side chain including L-Methionine, L-Leucine, L-Phenylalanine, L-Isoleucine, L-Tryptophan and L-Tyrosine. A significant activity was also observed for L-Cysteine. Over other amino acids, BjuLAAO had low catalytic activity (Fig. 1). This pattern shows a clear preference for hydrophobic residues with voluminous side chain and the affinity for residues with polar and/or small side-chains is significantly reduced or absent (Fig. 1). The LAAO from *Bothrops pauloensis* also showed preference for Met-, Leu-, Phe- and Ile- as substrates [7], suggesting that LAAOs encountered in *Bothrops* genus retain similar functions. In contrast, the LAAO from *Bungarus fasciatus* displayed higher specificity towards Tyr- and Asp- [19], whereas the bacterial LAAO (*Rhodococcus opacus*) has a very low substrate specificity hydrolyzing aromatic, aliphatic and polar amino acids [20].

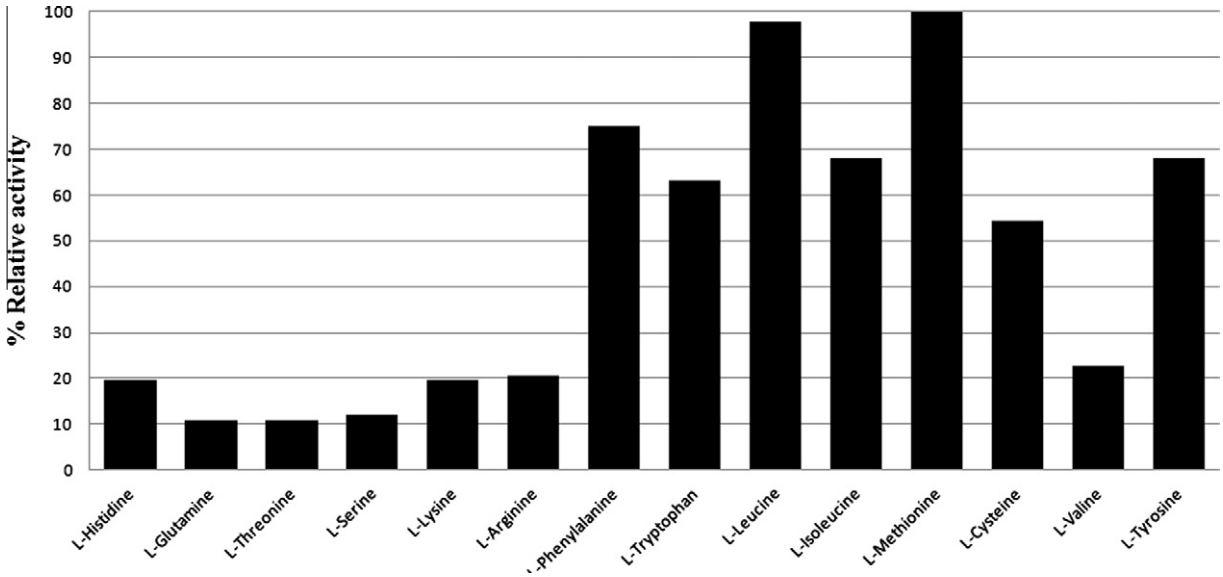

Fig. 1. Substrate specificity histogram. The LAAO activity was tested using different amino acids as substrate. We detected high activity for aromatic and hydrophobic amino acids, L-Methionine, L-Leucine, L-Phenylalanine, L-Isoleucine, L-Tryptophan and L-Tyrosine. The LAAO showed low catalytic activity for L-Histidine, L-Arginine, L-Valine, L-Threonine, L-Glutamine, L-Lysine and L-Serine.

Table 1
Data collection and refinement statistics.

Data statistics	
Temperature (K)	100
Radiation source	Brazilian Synchrotron Light Laboratory
Beamline	W01B-MX2
Wavelength (Å)	1.458 Å
Detector	MarMosaic 225 mm
Space group	$P2_1$
Unit-cell parameters (Å)	a = 66.38, b = 72.19, c = 101.53
Resolution range (Å)	30.0–3.10
R_{merge}[a] (%)	14.9 (31.1)
$\langle I/\sigma(I)\rangle$	5.16 (2.59)
Data completeness (%)	91.3 (79.6)
Redundancy	2.4 (2.3)
No. of measured reflections	16,155
Data analysis	
V_M (Å^3 Da^{-1})	1.84
Solvent content (%)	33.30
Molecules per asymmetric unit	2
Model refinement	
Resolution range (Å)	29.02–3.10
R_{free}	23.78
R_{factor}	17.61
Number of unique reflections	
Used in refinement	15,901
Number of	
Protein atoms	7606
Water molecules	17
FAD	2
R.m.s bond-length deviation (Å)	0.013
R.m.s bond-angle deviation (%)	1.667
Mean B-factor (Å^2)	31.40
Ramachandran plot analysis	
Most favored regions (%)	96.8
Allowed region (%)	2
Disallowed region (%)	1.2

[a] $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle(hkl)\rangle$ is the weighted average intensity for all observations I of reflection hkl.

3.2. The dimeric structure of BjsuLAAO

The BjsuLAAO structure was refined to a final R_{factor} of 17.6% and R_{free} of 23.8% at 3.1 Å resolution. Data-processing and refinement statistics are presented in Table 1. Analogously to other LAAOs [2,19–21], BjsuLAAO is comprised of three domains: FAD-binding domain (residues 35–72, 240–318 and 446–483), substrate binding domain (residues 5–25, 73–129, 231–239 and 319–445) and helical domain (130–230) (Fig. 2A). Sequence alignment among BjsuLAAO, *Agkistrodon halys pallas* and *Vipera ammodytes ammodytes* showed an average identity of 85% (Fig. 2B) with relevant amino acid substitutions in the FAD-binding domain and dimer interface (Fig. 2C). Other differences are limited to protein surface without any apparent contribution to protein function.

Electrostatic surface analysis of monomers of BjsuLAAO and LAAO from *Vipera ammodytes ammodytes* indicates that dimerization is mediated by Coulombic forces between substrate-binding domain (highly negatively charged) and FAD-binding domain (highly positively charged) (Fig. 3A and B). The crystallographic dimer that corresponds to the biological unit of BjsuLAAO was confirmed by in solution DLS analysis (Fig. S5) with a diameter of approximately 70 Å and an intermolecular interface of approximately 2081 Å^2. The key residues involved in dimer stabilization are K191, R317, H314, R317, S318, R300, R301, Y436, D376, D349, D210, D201, H320, T182, D205, D388, K186, R297 and H440.

In the structure of LAAO from *Vipera ammodytes ammodytes*, a zinc ion present in the tetrameric interface being coordinated by residues H75 and G279 was addressed as functionally and structurally important [2]. In the dimeric *B. jararacussu* and *Calloselasma rhodostoma* LAAO structures, no zinc ion was observed since the corresponding interface is absent. Furthermore, H75 is not conserved in BjsuLAAO being substituted by a Tyrosine. Biochemical data showed that the presence or absence of zinc ion did not interfere with the catalytic activity (results not shown). These observations strongly suggest that the zinc ion plays a role in tetramer stabilization, but not in LAAO activity.

3.3. A new FAD-binding mode with adenosyl moiety buried in the 62–71 loop

The FAD-binding domain is strategically located in the cleft formed by cofactor- and substrate-binding domains and is highly conserved in both prokaryotes and eukaryotes. The isoalloxazine ring of the FAD molecule is stabilized by extensive hydrogen bonds and Van der Waals contacts with residues G87, P88, M89, R90, L91, P92, G464, W465 and I466. Surprisingly, the adenosyl moiety was found in two different conformations in the asymmetric unit. In molecule B, this FAD portion presents a canonical binding mode mediated by Van der Waals contacts and being stabilized in this orientation by the ribose and di-phosphoryl groups that are tightly anchored to E63, Q71 and E457 side chains and backbone *N* atoms from M43 and S44 (Fig. 4A and C). In molecule A, the adenosyl group was found buried in the 62–71 loop representing a new

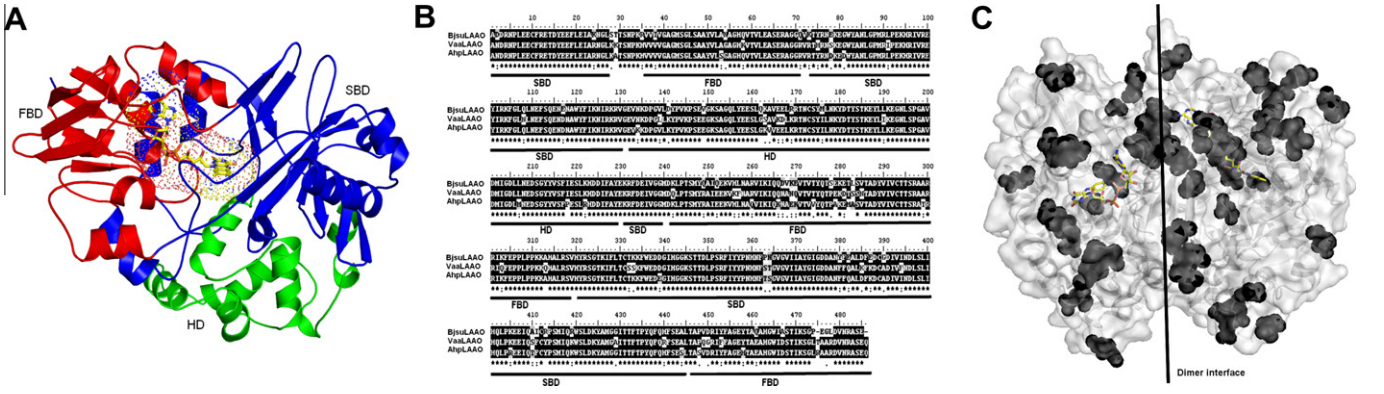

Fig. 2. BjsuLAAO structure and sequence alignment. (A) Structure comprising three domains: FAD-binding domain (FBD, red), substrate binding domain (SBD, blue) and helical domain (HD, green). The FAD molecule is represented in atom colors. (B) Sequence alignment among BjsuLAAO, *Vipera ammodytes ammodytes* and *Agkistrodon halys pallas* highlighting amino acid differences. (C) Residue substitutions in the dimeric structure shown in black. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

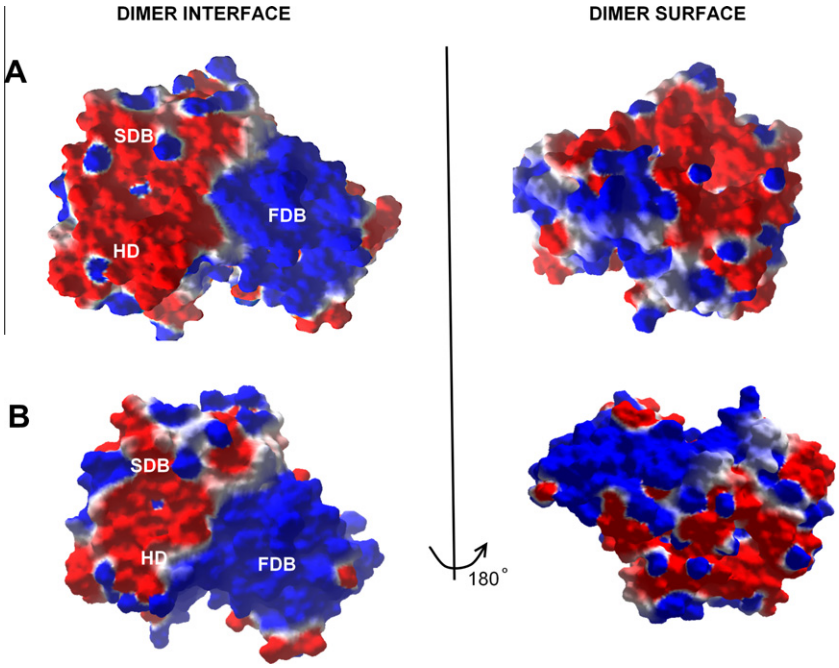

Fig. 3. Electrostatic surface analysis of BjsuLAAO (A) and LAAO from *Vipera ammodytes ammodytes* (B).

binding mode (Fig. 4B and C). In this novel orientation, the adenosyl group is stabilized by hydrogen bonds formed with residues E63, S65 and R67, and extensive hydrophobic contacts with most of the residues forming the 62–71 loop (Fig. 4B and C). This new conformation modifies the solvent accessibility to the FAD-binding domain increasing the cleft volume, previously occupied by the adenosyl group in the canonical-binding mode. Structural and energetic analyses indicated that the FAD molecule in the new orientation is equally stable to the canonical-binding mode suggesting to be biologically relevant for the activity of LAAOs.

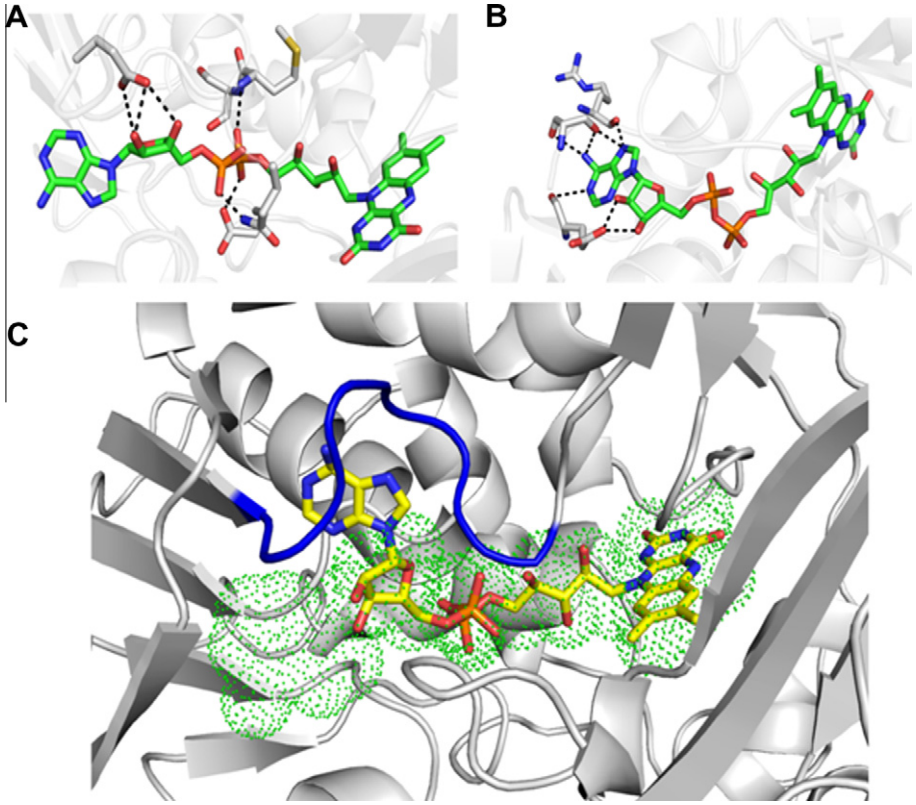

Fig. 4. FAD-binding mode in molecules B (A) and A (B) of BjsuLAAO crystal structure. (C) Superposition of FAD in molecule A (sticks in atom colors), and molecule B (green dots). The 62–71 loop is colored in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Active-site volume is related to LAAO substrate specificity

The BjsuLAAO activity is higher for aromatic and aliphatic amino acids with large side chains (Fig. 1). This result showed that BjsuLAAO is more specific than *C. rhodostoma* LAAO [22] that binds to a wide range of L-amino acids. Comparative structural analysis suggests that the small side-chain residues cannot adopt a specific or stable orientation in the active site of BjsuLAAO mainly due to the large cavity volume. In *C. rhodostoma* LAAO, the substitution of isoleucine at position 430 to a tryptophan makes the cavity volume smaller; implying in additional contacts of small side-chain residues with the active-site residues. Moreover, structural superposition of several snake venom LAAOs showed that the key residues for amino acid recognition are fully conserved including Y372, R90, W465, I430 and R322. This conservation indicates a highly specific function of snake venom LAAOs under the envenomation process.

In this work, a LAAO from *B. jararacussu* venom was purified and its functional and structural properties were determined. It was demonstrated that BjsuLAAO has higher specificity for hydrophobic residues and structural analysis indicates that the volume of active site is important for substrate specificity. Remarkably, a new FAD-binding mode equally stable to the classical-binding mode was observed, which could be biologically relevant for LAAO activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.129.

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