



ELSEVIER



CrossMark

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

Irreversible inactivation of snake venom L-amino acid oxidase by covalent modification during catalysis of L-propargylglycine[☆]

Jyotirmoy Mitra, Debasish Bhattacharyya*

Division of Structural Biology and Bioinformatics, CSIR-Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Kolkata 700032, India

ARTICLE INFO

Article history:

Received 14 December 2012

Received in revised form 28 January 2013

Accepted 28 January 2013

Keywords:

L-Amino acid oxidase
Crotalus adamanteus venom
Crotalus atrox venom
 L-Propargylglycine
 Irreversible inactivation
 Mechanism-based inhibitor

ABSTRACT

Snake venom L-amino acid oxidase (SV-LAAO, a flavin-enzyme) has attracted considerable attention due to its multifunctional nature, which is manifest in diverse clinical and biological effects such as inhibition of platelet aggregation, induction of cell apoptosis and cytotoxicity against various cells. The majority of these effects are mediated by H₂O₂ generated during the catalytic conversion of L-amino acids. The substrate analog L-propargylglycine (LPG) irreversibly inhibited the enzyme from *Crotalus adamanteus* and *Crotalus atrox* in a dose- and time-dependent manner. Inactivation was irreversible which was significantly protected by the substrate L-phenylalanine. A Kitz–Wilson replot of the inhibition kinetics suggested formation of reversible enzyme–LPG complex, which occurred prior to modification and inactivation of the enzyme. UV–visible and fluorescence spectra of the enzyme and the cofactor strongly suggested formation of covalent adduct between LPG and an active site residue of the enzyme. A molecular modeling study revealed that the FAD-binding, substrate-binding and the helical domains are conserved in SV-LAAOs and both His223 and Arg322 are the important active site residues that are likely to get modified by LPG. Chymotrypsin digest of the LPG inactivated enzyme followed by RP-HPLC and MALDI mass analysis identified His223 as the site of modification. The findings reported here contribute towards complete inactivation of SV-LAAO as a part of snake envenomation management.

© 2013 The Authors. Published by Elsevier B.V. on behalf of Federation of European Biochemical Societies. All rights reserved.

1. Introduction

L-amino acid oxidase (LAAO), a dimeric enzyme containing non-covalently bound FAD as cofactor, is widely present in eukaryotic and prokaryotic organisms [1]. They catalyse stereospecific oxidative deamination of L-amino acids to α-keto acid via α-imino acid intermediate along with generation of ammonia and hydrogen peroxide (Fig. 1). These enzymes exhibit a marked preference for hydrophobic amino acids like phenylalanine, tryptophan, tyrosine and leucine as substrate [2,3]. This substrate preference originates from the binding of hydrophobic side chains of amino acids with the enzyme. The catalysis is proposed to follow either of the two different mechanisms: a carbanion pathway in which the proton is transferred to the FMN/

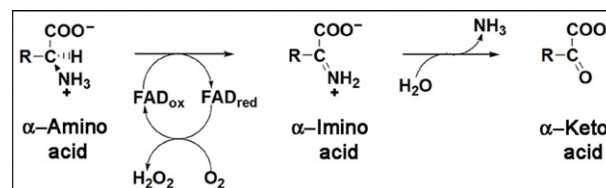


Fig. 1. Catalytic oxidation of L-amino acid oxidase. The first half reaction involves the conversion of FAD to FADH₂ with concomitant oxidation of the amino acid to an imino acid. Another oxidative half reaction completes the catalytic cycle by reoxidizing the FADH₂ with oxygen, producing hydrogen peroxide. The unstable imino acid is then hydrolyzed by the enzyme bound water to form the final product keto acid.

[☆] This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abbreviations: CHD, 1,2-cyclohexanedione; DEPC, diethylpyrocarbonate; FAD, flavin adenine dinucleotide; Gdn-HCl, guanidine hydrochloride; LAAO, L-amino acid oxidase (EC. 1.4.3.2); LPG, L-propargylglycine; L-Phe, L-phenylalanine; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; TNBS, trinitrobenzene sulfonic acid.

* Corresponding author. Tel.: +91 33 2499 5764; fax: +91 33 2473 5197.

E-mail address: debasish@iicb.res.in (D. Bhattacharyya).

FAD cofactor from the α-carbon atom of the substrate leaving a negative charge followed by two electron transfer. Alternately, a hydride transfer pathway in which an alpha hydrogen atom is transferred as a hydride ion carrying two electrons simultaneously. Recent structural and mechanistic studies on D-amino acid oxidase (DAAO) support the hydride transfer mechanism [4].

LAAOs isolated from snake venoms are the best characterized members of this enzyme family. High abundance of the enzyme in

snake venom causes potential toxicity, as it induces impairment of platelet aggregation together with necrotic and apoptotic cell death [2,5,6]. This effect is primarily attributed to the production of high concentration of localized H_2O_2 [7,8]. LAAO from *Crotalus adamanteus* and *Crotalus atrox* can associate specifically with mammalian endothelial cells possibly through the glycosylation site of the enzyme [7,9]. Such association and interaction of LAAO with bacterial cell was observed in case of *Trichoderma harzianum* ETS323 [10]. It is predicted that the glycan moiety of LAAO is used in docking the enzyme to the cell surface, thus enhancing the localization of H_2O_2 [7,9]. This enzyme share sequence similarity with human monoamine oxidase (MAO) and may involved in allergic inflammatory response [11]. Recent crystallographic studies of LAAO from *Calloseasma rhodostoma* complexed with L-Phe and o-aminobenzoic acid reveal that the catalytic site of each subunit of the dimeric enzyme is composed of three parts: a FAD-binding domain, a substrate-binding domain and a helical domain [12,13]. High degree of structural similarity of these three domains of LAAO from *C. rhodostoma*, *Bothrops jararacussu* and *Bothrops moojeni* was observed through comparative sequence homology and molecular modeling [14]. Also LAAO from *C. rhodostoma* shares 83% sequence identity with that of both *C. adamanteus* and *C. atrox*. Overall, snake venom LAAOs are highly conserved with respect to their structure, function and substrate specificity.

A variety of mechanism based inhibitors have been reported for a number of flavoenzymes [15,16]. These inhibitors can inactivate the enzymes either by modifying the cofactor or a reactive amino acid residue during catalytic turnover [17–24]. Success of such inhibitors has generated an increasing interest towards designing of suicidal substrate for LAAO for partial neutralization of venom toxicity. Earlier reports indicate that L-propargylglycine (LPG) acts as a substrate of lower affinity and not an inhibitor of LAAO from *C. adamanteus* whereas D-propargylglycine acts as an inhibitor of D-amino acid oxidase (DAAO) [25–27]. Inactivation of LAAO by LPG was not realized, either due to the low concentration of LPG applied or the kinetic of inactivation was followed for a short time. Here, we report that LPG irreversibly inactivates LAAO by modifying active site His223 during its catalytic turnover. Thus it acts as a mechanism-based inhibitor. Similar results were also observed with LAAO from *C. atrox* venom.

The working hypothesis of the current observation is that there are multiple substrate/inhibitor binding sites in the catalytic funnel of LAAO of which one is responsible for catalytic turnover. It is believed that LPG at low concentration binds exclusively at the catalytic site and undergoes enzymatic conversion. But at higher concentration, LPG binds at multiple sites including the catalytic site of LAAO. Binding of LPG at noncatalytic sites probably induces conformation change of the enzyme that leads to covalent modification of the enzyme with LPG undergoing conversion at the catalytic site leading to a dead end complex. This assumption is based on the crystal structures of LAAOs where existence of multiple substrate/inhibitor binding sites in the catalytic funnel is evident [12,13]. The purpose of this study is to gain insight for designing specific and potent inhibitors of LAAO.

2. Results

2.1. Oxidation of LPG by LAAO

Unless mentioned otherwise, LAAO used in this work refers to the enzyme from *C. adamanteus*. LPG, a propargyl derivative of L-glycine has all the features that are essential for becoming a substrate of LAAO, e.g., amino and carboxylic groups along with a hydrophobic side chain. LPG up to 500 μ M acts as a substrate of LAAO for at least 180 min when monitored at 320 nm (Fig. 2A). The initial rates and the amounts of product formed by 180 min when it is assumed that the reaction is complete, show linear dependency with LPG concentration (Fig. 2B and C). Since no inactivation was observed during the course of turnover, it is clear that irreversible enzyme–inhibitor adduct is

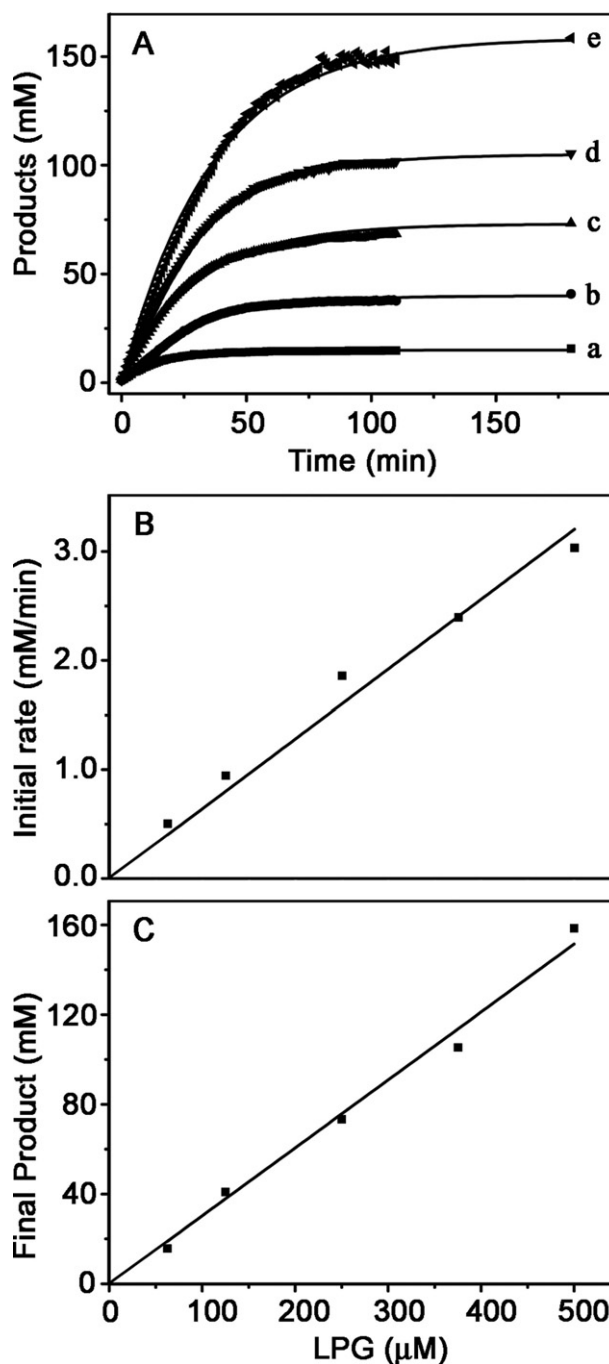


Fig. 2. (A) Time course of oxidation of (a) 62.5, (b) 125, (c) 250, (d) 375 and (e) 500 μ M of LPG by 81.22 nM of LAAO. Experimental conditions have been mentioned in the text. (B) Linear dependence of the initial rate and (C) product formation at 180 min, when the reactions was assumed to be complete with the concentration of LPG. R^2 (regression coefficient) values are 0.992 and 0.996, respectively.

not formed under that concentration of LPG. Therefore, it is assumed that the absorbance at 320 nm is exclusively contributed by the LPG oxidation product 3-amino-5-methylene-5H-furan-2-one and not by the adduct that also absorbs at 320 nm (discussed later).

In order to compare the affinity of the substrates L-Phe and LPG, Michaelis–Menten plots were constructed. While measuring the initial rates, the reactions were followed for 120 s where linear increment of product formation was observed. Dependence of initial

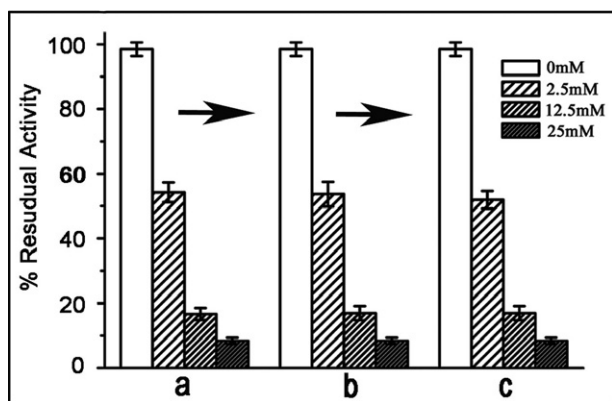


Fig. 3. Dependence of irreversible inactivation of LAAO on the concentration of LPG. (a) Residual activity of LAAO after incubation with 0–25 mM of LPG at 4 °C for 24 h. (b) Activity of the enzyme preparations of set (a) after passing through Sephadex G-50 'Spin column' to remove unreacted LPG and its catalytic products. (c) Activity of the enzyme preparation of set (b) after incubation at 4 °C for 24 h allowing time dependent dissociation of enzyme–inhibitor adduct, if any. Activity of the enzyme before passing through 'Spin-column' is taken as 100%.

rates as a function of substrate concentration resulted in a rectangular hyperbolic profile (Supplementary Fig. S1A and B). Corresponding double reciprocal (Lineweaver–Burk) plots show good linearity (Supplementary Fig. S1, insets). The kinetic parameter derived from Michaelis–Menten plot showed that L-Phe ($K_M = 37.82 \pm 2.82 \mu\text{M}$, $k_{cat} = 6.27 \pm 0.1 \text{ s}^{-1}$) is more susceptible to oxidation by LAAO than LPG ($K_M = 7.95 \pm 0.43 \text{ mM}$, $k_{cat} = 9.62 \pm 0.18 \text{ s}^{-1}$) ($n = 4$). However L-glycine was not oxidized by LAAO (data not shown). These results suggest that the propargyl group in LPG makes it a better substrate than L-glycine but significantly poorer as compared to L-Phe (Table 1).

2.2. Irreversible inactivation of LAAO by LPG

Incubation of LAAO (0.5 U, 81.22 nM, 0.812 nmol) in presence of 25 mM LPG (25 μmol) at 30 °C for 2 h resulted in reduction of its oxidase activity by >90%. An additional application of 25 mM of LPG at the end of 2 h eventually led to complete inactivation of the enzyme by an hour. Under identical conditions and in absence of LPG, the enzyme is inactivated at most by 10%. To exclude product inhibition, the oxidation products were isolated by ultrafiltration through 30 kDa cut-off filter membranes and were incubated with LAAO. No product inhibition was observed. Irreversible nature of the inhibition was ascertained by incubating the enzyme in presence of 2.5–25 mM of LPG at 4 °C for 24 h whereby the residual activity was reduced to approximately 10%. The inactive enzyme was passed through 1-ml Sephadex G-50 'Spin-column' to remove excess inhibitor and the oxidation products [28]. Oxidase activity was measured immediately by taking an aliquot and rest of the sample was kept at 4 °C for 24 h to allow slow dissociation of the enzyme–inhibitor complex, if any, in absence of excess inhibitor. In either case, lost activities could not be recovered. In absence of LPG, the enzyme was completely stable (Fig. 3). This data suggest that the substrate analog LPG irreversibly inhibits LAAO and that through nondissociable association. The situation that LPG acts as a substrate of LAAO and also irreversibly inhibits LAAO has been schematically presented in Fig. 4.

2.3. Protection of inactivation by substrate

Incubation of LAAO with LPG at the same molar ratio as stated above at 4 °C for 24 h resulted in 90% loss of activity. To determine whether inactivation occurs during catalysis and at the active site, the

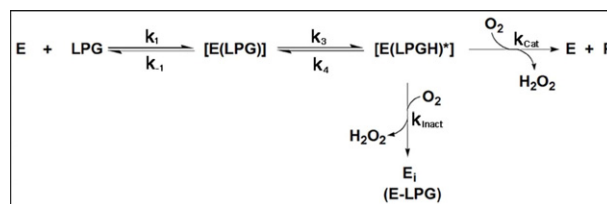


Fig. 4. Presentation of the mechanism of inactivation of LAAO, presented as E, by the suicidal substrate LPG. The ratio k_{cat}/k_{inact} defines the partition ratio and it denotes the inhibitor potency of the inhibitor, as it expresses the extent of bifurcation of a reaction towards product formation and inactivation.

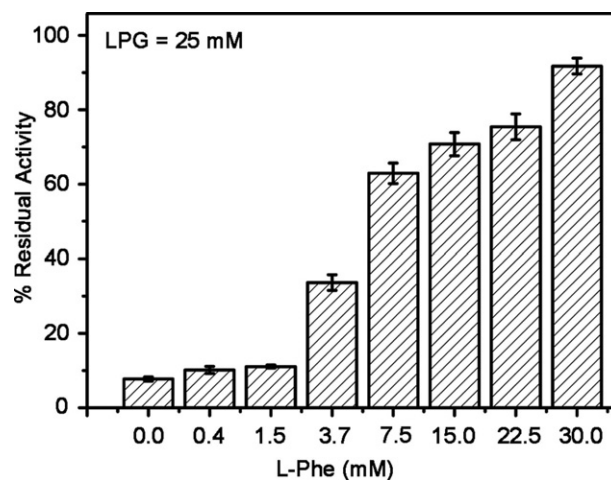


Fig. 5. Protection of LAAO activity with concentration of L-Phe in presence of 25 mM of LPG. Activity of the enzyme incubated with 0.38 mM L-Phe is considered as 100%.

incubation was carried out in presence of 0–30 mM of L-Phe substrate. Activity of these enzyme preparations were measured after extensive dialysis at 4 °C. It was observed that with increasing concentration of L-Phe, 90% of the enzyme activity could be protected (Fig. 5). This suggests that LPG compete with the substrate L-Phe during inactivation of LAAO.

2.4. Inactivation kinetics of LAAO with LPG

Irreversible inactivation of LAAO was further analyzed after preincubation of the flavoenzyme with 5–25 mM of LPG, followed by dilution into assay buffer to determine residual activities. The enzyme (0.5 U, 81.22 nM, 0.812 nmol) showed both concentration and time-dependent inhibition with respect to LPG. The kinetics of inactivation (k_{obs}) was determined from the linear dependencies with logarithm of percent residual activity (Fig. 6A). To determine the Kitz–Wilson kinetic parameters, the observed rate constant at various inhibitor concentrations were fitted to Eq. (1) [29].

$$\frac{1}{k_{obs}} = \frac{1}{k_{inact}} + \frac{K_I}{k_{inact}} \left(\frac{1}{[I]} \right) \quad (1)$$

where K_I is the apparent inhibitor concentration required to reach half-maximal rate of inactivation, k_{inact} is the maximal rate of enzyme inactivation and $[I]$ is inhibitor concentration. Kitz–Wilson replot of the observed rate constants yielded $k_{inact} = 0.0925 \pm 0.0025 \text{ min}^{-1}$ and $K_I = 7.49 \pm 0.46 \text{ mM}$. The straight line in Kitz–Wilson replot intersects the ordinate at 10.82 min indicating formation of dissociable enzyme–inhibitor complex that occurs before inactivation (Fig. 6B). In case the line passes through the origin, that indicates absence of a reversible complex formation and inactivation is a direct consequence of enzyme inhibitor collision. This data suggest that LPG acts

Table 1
Kinetic parameters of LAAO.

Venom source	L-Phenylalanine		L-Propargylglycine			
	k_{cat} (s^{-1}) ^a	K_M (μM) ^a	k_{cat} (s^{-1})	K_M (mM)	k_{inact} (min^{-1}) ^b	K_I (mM) ^b
<i>C. adamanteus</i>	6.27 ± 0.10	37.82 ± 2.82	9.62 ± 0.18	7.95 ± 0.43	0.0925 ± 0.0025	7.49 ± 0.465
<i>C. atrox</i>	6.78 ± 0.06	36.01 ± 1.50	7.75 ± 0.50	11.78 ± 2.44	0.0825 ± 0.0067	9.69 ± 1.22

^a k_{cat} and K_M were obtained from Michaelis–Menten plot constructed from the initial kinetics of 120 s.

^b k_{inact} and K_I were determined using Eq. (1) as described in the text.

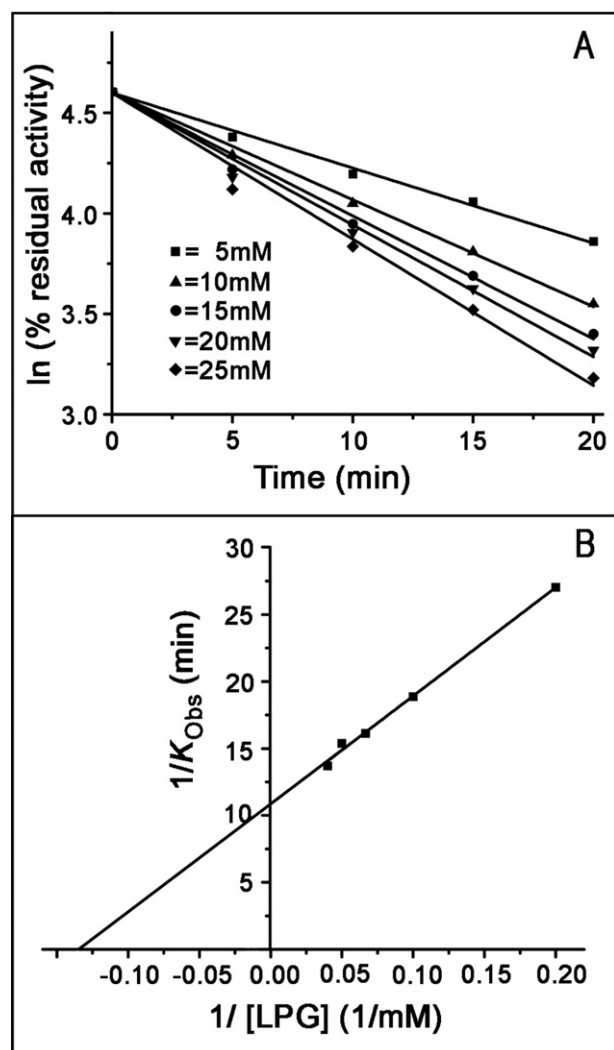


Fig. 6. (A) Time dependent inactivation of LAAO with variable concentration of LPG at 30 °C. (B) Kitz–Wilson replot from the adjacent kinetic data correlating the inverse of k_{obs} with the inverse of LPG concentration.

as a mechanism-based inhibitor of LAAO. It may be noted that from the K_I predicted above, approximately 15 mM of LPG is needed for complete inactivation of LAAO whereas 25 mM of LPG is required. A possible explanation is, during irreversible modification of LAAO, a portion of LPG acts as substrate leading to gradual decrease of LPG concentration affecting the kinetics of inactivation. Further, because of irreversible modification of the enzyme, concentration of active enzyme decreases too with time leading to slower rate of inactivation.

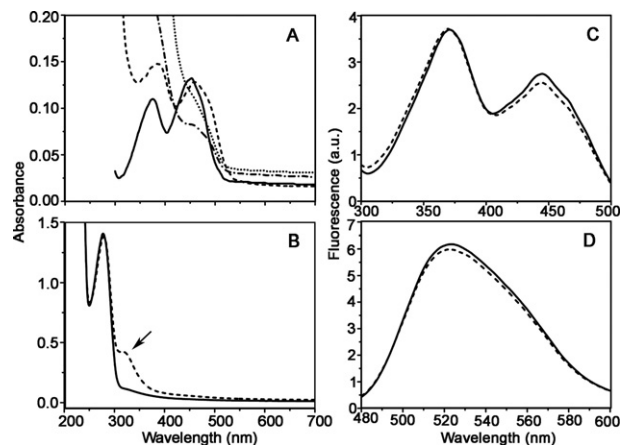


Fig. 7. (A) UV–visible spectra of FAD (13 μM) (—), LAAO dialyzed against buffer (---), LAAO inactivated by LPG before (.....) and after dialysis (----). LAAO concentration was 0.5 U/ml. (B) UV–visible spectra of apo-LAAO (—) and LPG inactivated apo-LAAO (---). The later shows a distinct peak at 320 nm marked by arrow. (C) Emission and (D) excitation spectra of FAD from control LAAO (—) and LPG inactivated LAAO (---). These spectra are identical with that of reference FAD that had not been included for clarity.

2.5. Spectral analysis of inactivated complex

LAAO after complete inactivation by LPG was extensively dialyzed to remove unreacted LPG and its oxidation products. Fig. 7A and B shows the UV–visible spectra of the inactivated enzyme. The control enzyme when dialyzed under identical conditions shows spectrum that is similar to FAD in its oxidized form as reported earlier [31]. The inactivated enzyme shows the presence of an enzyme-bound chromophore, which has strong absorbance between 300–400 nm. To remove the chromophoric adduct from the enzyme, the complex was denatured with 8 M Gdn-HCl to dissociate the adduct as well as the flavin and was followed by ultrafiltration to remove the small molecules [30]. The spectrum of the apoenzyme prepared from the inactivated complex has a distinct absorption at 320 nm (Fig. 7B). Similar observation was reported for oxidation of D-propargylglycine by D-amino acid oxidase [27]. This indicates association of the chromophore with enzyme's active site residue and not with the cofactor. The characteristic fluorescence spectra of the released FAD was identical with the FAD released under similar condition from unmodified enzyme and reference FAD (excitation spectra, em: 525 nm, ex: 300–500 nm, Fig. 7C; emission spectra, ex: 450 nm, em: 480–550 nm, Fig. 7D). The $\lambda_{max,ex}$ = 360 and 450 nm and the $\lambda_{max,em}$ = 525 nm were identical for FAD released from LPG inactivated enzyme and the control active enzyme. The spectral data collectively indicate that inactivation of the enzyme by LPG is a consequence of covalent modification that does not affect the FAD cofactor.

2.6. Sequence analysis and modeling study

Multiple sequence alignment of LAAOs from *C. adamanteus* with *C. atrox*, *Agkistrodon halys*, *Vipera ammodytes ammodytes* and *C. rhodostoma* using Clustal W2.1 showed high homology (>83% sequence identity) (Fig. 8A). Homology models of LAAO from *C. adamanteus* generated from 'Swiss model' and 'CPH model' servers were exactly the same as visualized by structural alignment using PyMol software [32]. Furthermore superimposition of these model with that of *C. rhodostoma* LAAO crystal structure reveals high degree of structural similarity between FAD-binding, substrate-binding and helical domains of these proteins. Pawelek et al. [12] have shown that Arg90, Glu209, His223, Asp224, Tyr372 and Gly464 are responsible for contact with three *o*-aminobenzoate inhibitor molecules observed in the catalytic funnel. These residues direct the access of the substrate to the active site and they superimpose on the same residues of the *C. adamanteus* LAAO model. These demonstrate similar catalytic site orientation between *C. adamanteus* and *C. rhodostoma* LAAOs. So *C. adamanteus* LAAO model was aligned with the crystal structure of LAAO from *C. rhodostoma* (PDB ID: 2IID). Since, LPG acts as a substrate of LAAO, it must transfer α -hydrogen atom to FAD analogous to L-Phe reaction. So LPG was manually docked at the position of the bound substrate L-Phe [13]. While docking, the spacial orientations of the functional groups (amino and carboxyl group) and the α -carbon atom were kept unaltered as that of L-Phe in *C. rhodostoma* LAAO crystal structure (Fig. 8B). As predicted by Moustafa et al. [13] the amino group and the carboxyl group of the substrate analog LPG are in a position to form H-bond with Gly464 and Arg90 respectively. The side chain of the amino acid analog is extended away from the cofactor and is accommodated in a sub-pocket composing Ile430, His223 and Arg322 (Fig. 8D). Noteworthy observation is that His223 and Arg322 were within a distance of 3.7–7.0 Å from the C2 atom of the acetylenic side chain of LPG and thus, they are prone to be modified by LPG.

2.7. Modification reaction

Modification of the histidine and arginine residues of LAAO by the group specific reagents DEPC and CHD resulted in complete inactivation (Fig. 9). This suggests that both of the residues are involved in catalysis. Hydroxylamine, HCl treatment of the enzyme resulted in loss of 60% of activity. So reversal of histidine modification was not performed. Complete protection of activity in case of histidine modification was observed in presence of 7.7 mM of L-Phe whereas in presence of 1 mM LPG, 15–20% activity was protected. Insignificant protection of activity was observed in case of arginine modification. Modification of lysine residues by TNBS had no effect on enzyme activity (Fig. 9). These results suggest that inactivation due to modification of active site residues can only be protected in case of histidine. Also as the substrate protects inactivation of the enzyme by LPG, it suggest a possible participation of His223 in the adduct formation.

2.8. Covalently modified of His-223

Covalent modification of the enzyme by LPG occurs probably through a nucleophilic attack by an active site residue of the enzyme to the C3 atom of LPG (Fig. 10). Intact mass of LAAO from *C. adamanteus* was provided by MALDI-TOF MS as $57,476 \pm 100$ Da (Supplementary Fig. S2). The enzyme inactivated by LPG is expected to have a mass higher by 112 Da due to covalent adduct formation. But, it could not be detected as the instrument has a 100 Da mass tolerance in the Mw range used. The cofactor isolated from the active and LPG inactivated enzyme showed an identical mass of 784 Da in the negative ion mode (Supplementary Fig. S2, inset). This supports the absorption and fluorescence data presented before.

The covalent adduct so formed has strong absorption at 320 nm due to the extended conjugated double bond structure (Fig. 10). This

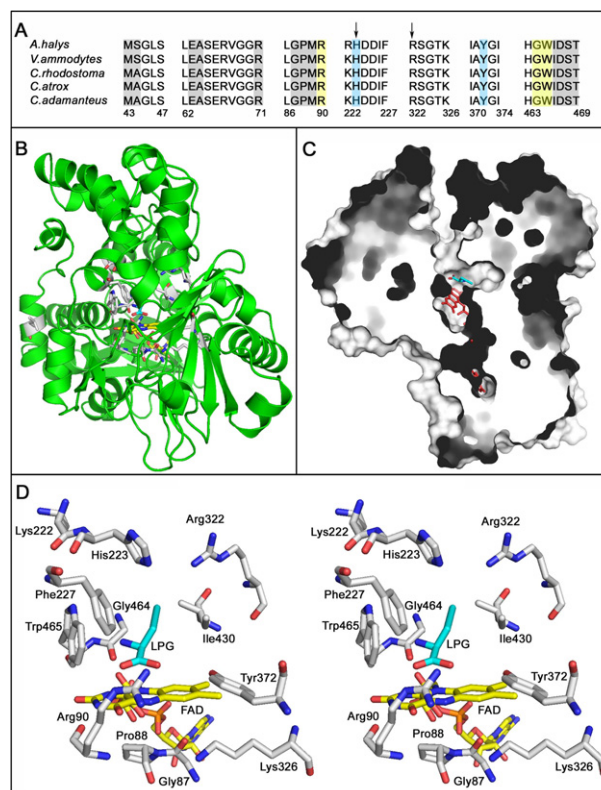


Fig. 8. (A) Sequence alignment of the catalytic region of LAAOs from different venom source, indicating conserved positions of histidine and arginine residues (marked by arrows). The FAD and substrate binding residues are highlighted in light black and blue respectively while yellow shade represents both substrate and FAD binding residues. (B) Homology model of *C. adamanteus* LAAO obtained from CPH model 3.0 online server. The catalytic site residues have been shown and they are deeply buried within the enzyme. (C) Molecular surface representation of LAAO is used to help visualization of the catalytic funnel where LPG being docked at the position of normal substrate, maintaining a distance of 3.5 Å between α -C atom of LPG (cyan) and flavine (red). (D) Stereo view of the catalytic site of LAAO showing the environment of LPG. The substrate LPG is shown in cyan and the FAD in yellow. His223 and Arg322 that are susceptible to modification are present in the side-chain binding sub-pocket and nearest to the acetylenic moiety of LPG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

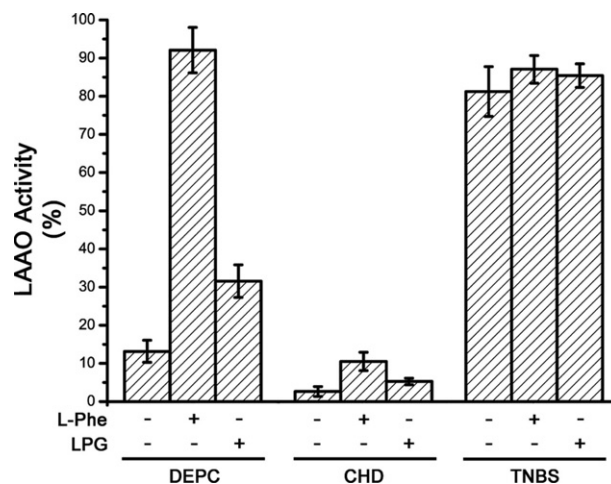


Fig. 9. Residual activity of LAAO (%) after modification with DEPC, CHD and TNBS in presence and absence of L-Phe or LPG.

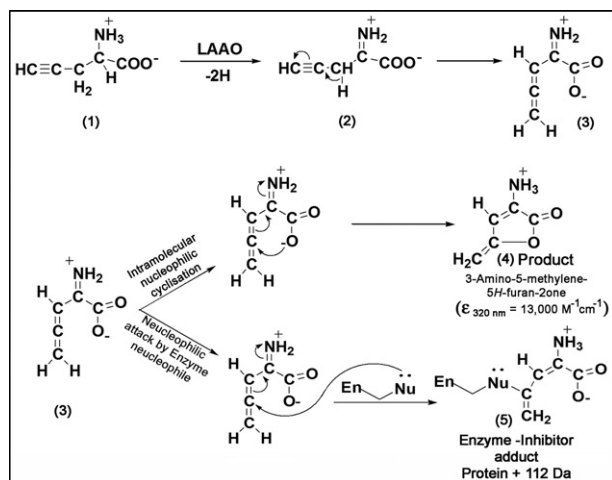


Fig. 10. Proposed mechanism to explain the catalytic and suicide inactivation pathway of L-tryptophan oxidase by LPG. LPG (1) is oxidised by LAAO to the acetylenic imine intermediate (2). The intermediate (2) can rearrange to the allenic imine intermediate (3), which either cyclises through intramolecular nucleophilic attack to give the product 3-amino-5-methylene-5H-furan-2-one (4) or undergo nucleophilic attack by a nucleophile present at the active site of the enzyme to form the inactive enzyme-inhibitor adduct (5).

attribute made it possible to locate the residue in LAAO modified by this inhibitor. LAAO, inactivated irreversibly by LPG, was extensively dialysed and subjected to α -chymotrypsin digestion. Use of trypsin as the protease for the identification of the LPG modified peptide was unsuccessful (discussed later). Application of the digest to RP-HPLC under water-acetonitrile gradient led to separation of a number of peptides when followed at 220 nm (Fig. 11). When the same chromatogram was followed at 320 nm, elution of a major sharp peak of retention time (R_t) 29.4 min was observed (Fig. 11A, lower inset). Under identical conditions of HPLC with a control digested sample, no component with absorption at 320 nm was eluted (Fig. 11A, upper inset). The isolated peptide when subjected to MALDI mass analysis yielded m/z of 884.89 Da ($[M+H]^+$) (Fig. 11B). This peak has been assigned to the peptide KH*DDIF (773 Da + 112 Da for the LPG adduct marked as *). MSMS analysis of this peptide shows mass ion of 166.22, 284.22, 385.93 and 499.34 Da which correspond to F, KH, HDD and HDDI peptide, respectively (Supplementary Fig. S3). Further, digestion of the unmodified enzyme with chymotrypsin did not yield a peptide of 884.89 Da. Also LAAO from *C. atrox* after inactivation by LPG and digestion by chymotrypsin showed a peak of $R_t = 29.4$ min in RP-HPLC when followed at 320 nm and that also yielded m/z value of 884.89 Da in mass analysis (Supplementary Fig. S4). These results indicate that modification by LPG involves His223 residue sparing the FAD moiety.

3. Discussion

Substrate analogues as mechanism-based inhibitor have received considerable interest for several flavoenzymes as their catalytic site contain reactive flavin in addition to functional amino acids [15,16]. Irreversible inhibition of flavoenzymes with acetylenic substrate analogs mostly reveals covalent adduct formation through N5 or C4a atom of the isoalloxazine moiety of FAD or FMN [33–36]. In case of LAAO from *C. adamanteus*, LPG acts as a weak substrate at low concentration (Fig. 2A). However, at higher concentration it acts as an irreversible inhibitor. Since the catalytic funnel has multiple sites to accommodate substrate/inhibitor molecules, it is expected that at high concentration of LPG, these sites get saturated [12,13]. Probably

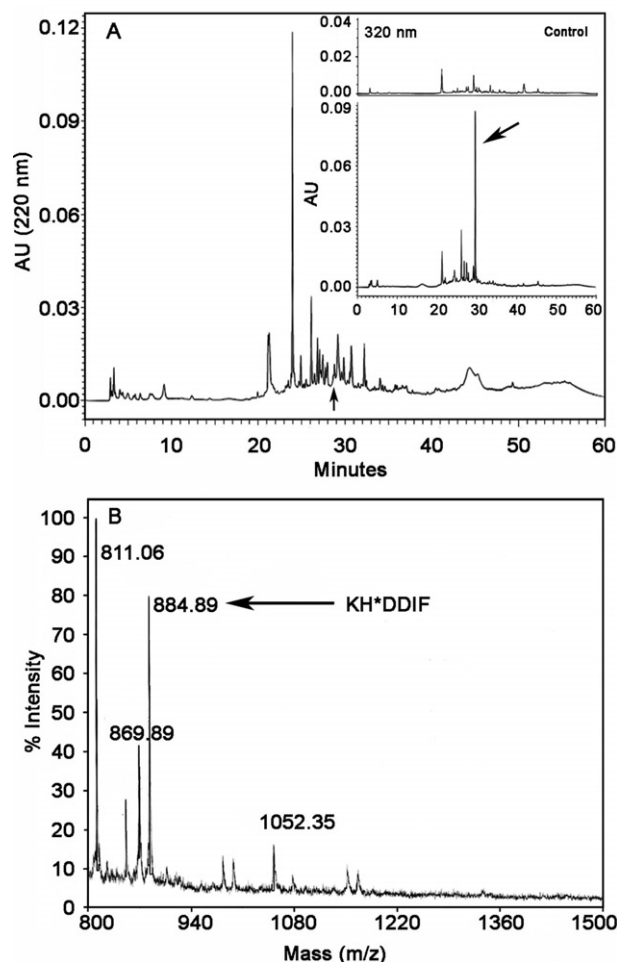


Fig. 11. (A) RP-HPLC chromatogram of chymotryptic digest of LAAO inactivated by LPG monitored at 220 nm. Inset. The same chromatogram of control LAAO (upper) and that of LPG inactivated LAAO (lower) respectively monitored at 320 nm. The peak of R_t 29.4 min marked by arrow corresponds to the LPG modified peptide. (B) MALDI mass spectrum shows m/z of 884.89 Da, which correspond to the modified peptide KH*DDIF where * denotes LPG adduct. The MSMS spectrum of this marked peptide is shown in Supplementary Fig. S4.

this induces conformational change of the enzyme that allows covalent interaction of the propargyl group with the nucleophilic amino acid residues of LAAO (Fig. 3). The structure of LPG as compared to the substrate L-Phe, indicates that it may act as an active site directed inhibitor. It is clear that the said LAAO-LPG reversible complex undergoes two simultaneous reactions. One of them leads to product formation while the other leads to an inactive adduct (Fig. 4). The nature of binding of LPG as inhibitor is covalent as the enzyme-inhibitor adduct could not be dissociated from the inactive enzyme after passing through 'Spin-column'. Further, neither the enzyme-inhibitor complex did undergo time dependent dissociation nor the inhibitory moiety could be separated from the enzyme under denaturing conditions (Figs. 3 and 7).

It is interesting to note that up to 0.5 mM, LPG acts faithfully as a substrate of LAAO and not as an inhibitor reflecting its predominance substrate like character. The hydrophobicity imparted by the propargyl group makes LPG a suitable substrate of LAAO. Though the K_M of LPG is 210 fold higher than L-Phe, Michaelis-Menten relation and Lineweaver-Bark dependency were well followed (Supplementary Fig. S1). So, at low concentration of LPG, the fate of the acetylenic intermediate is to cyclise leading to product formation [26]. Then

the question arises, what promotes LPG to act as an inhibitor at 5–25 mM? Similar requirement of high concentration of inhibitors for effective inhibition is known for monomeric sarcosine oxidase [37]. The crystallographic structure of LAAO shows that its catalytic site is funnel like where the substrate enters into a pocket and the products leave through the other end [13]. The catalytic region has multiple substrate binding sites of variable affinities [12]. It is speculated that at high concentration, these sites get saturated with LPG, which affects the geometry of the catalytic funnel, leading to a decrease in the distance between the catalytic site and the irreversible modification site. The modification site is meant to be the sub-pocket of the enzyme where the side-chain of the substrate amino acid or the analog is accommodated. His223, present in this sub-pocket, is believed to interact with LPG (discussed below). The Kitz–Wilson replot also predicts formation of a reversible enzyme–inhibitor complex followed by irreversible inactivation (Fig. 6). In presence of high concentration of L-Phe, inactivation of LAAO by LPG is prevented by 90% indicating competition between the substrate and the inhibitor (Fig. 5).

Based on the evidence presented here, dual character of LPG as substrate and inhibitor can be predicted from the chemistry of the reaction. In case of L-Phe, the oxidation product is an unstable α -imino acid, which is hydrolyzed to α -keto acid. Spectroscopic and mass data show that FAD of the LPG inactivated LAAO is in its oxidized form whereas a covalent enzyme adduct is formed. Therefore, an active site amino acid residue gets modified. The reaction of LPG with LAAO is likely to proceed as described in Fig. 10. First, LPG (1) is enzymatically converted to the acetylenic imine intermediate (2) with concomitant reduction of FAD to FADH₂ (resembling Fig. 1). Second, (2) undergoes rearrangement to an allenic imine intermediate (3). This intermediate (3) undergoes nucleophilic attack either by the carboxyl group of LPG itself or a nucleophilic residue of the enzyme. The former leads to product formation (4) through cyclisation [26] while the later results in irreversible modification of the enzyme (5) similar to other mechanism-based inhibitions [38–42].

While supporting the proposed mechanism of inhibition, comparison of the structure of LAAOs from different venoms were done. Complete conservation of the substrate and FAD binding domains of LAAO from *C. adamanteus* with that of *C. rhodostoma* was observed from sequence alignment and homology modeling (Fig. 8A and B). The catalytic site of LAAO containing FAD is deeply buried in a 25 Å long funnel-like entrance within the enzyme body. The funnel wall contains both hydrophilic and hydrophobic residues to guide the substrate or its analogue in a way to place the α -C atom within an average of 3.5 Å from the flavin N5 atom for an effective hydride transfer [12,13,43]. The homology model was structurally aligned with the crystal structure (PDB ID: 2IID) and LPG was manually aligned at the position of L-Phe maintaining a distance of ~3.5 Å from flavin N5 [13]. It was observed that the acetylenic side chain of LPG is within 3.7–7.0 Å from His223 and Arg322 residues located in the side chain binding sub-pocket (Fig. 8D). The imidazole N-atom of histidine is in the deprotonated form ($pK_a \sim 6.04$) as the pH used for the inactivation reaction was 7.5. The side chains of Arg and Lys have pK_a of ~12.48 and ~10.79 respectively. So His223 seems to be in a better position to form a covalent adduct with the LPG intermediate. Lys and Ser, which are susceptible to such modification are also present in the catalytic funnel but are not suitable targets due to greater distances. The role of His, presumably the 223 residue, in adduct formation was verified from amino acid modification reactions. Inactivation of the enzyme after histidine and arginine modification indicated involvement of these residues at the catalytic site. In case of only histidine, prevention of inactivation by the substrate supports that His223 as the most probable target for LPG modification.

Once confirmed that FAD is not the site of modification by LPG, attempts were made for fingerprinting of the inactive enzyme by MALDI-MS after tryptic digestion. The result was compared with untreated LAAO. Unfortunately, peptide peaks of mass difference 112

Da for the LPG modified fragment could not be detected with full confidence. This is possibly due to the low abundance of modified peptide. In the amino acid sequence of LAAO, the K222–H223 and K231–R232 bonds are resistant to trypsin digestion, due to modification of H223 and repeat basic amino acid sequence. Thus the modified peptide should be confined between K191 and K252 of 6821 Da. This peptide may be too large for an efficient ionization. So, a different proteolytic enzyme was selected to identify the site of modification. The LAAO from both *C. adamanteus* and *C. atrox* was inactivated with LPG, dialyzed extensively and subjected to chymotrypsin digestion to yield small peptides. The modified peptide was then purified using RP-HPLC and finally sequence of the isolated peptide was ascertained as KH*DDIF by mass analysis where * indicates LPG modification (Fig. 11A and B, Supplementary Figs. S3 and S4).

The finding that LPG is an irreversible inhibitor of LAAO shows a direction for developing more potent inhibitors of the enzyme that is required in snakebite management. Catabolism of free L-amino acids in blood by LAAO after snake envenomation, particularly at the site of bite, is the major route to peroxide generation that leads to cell apoptosis and tissue damages. Hydrophobic amino acids content in human blood plasma are in the range of 60–120 μ M after normal diet which is far less than that required for the protection of LPG reaction [44,45]. Compounds with similar structure as of LPG that have higher k_{inact} than k_{cat} values would hold good therapeutic values for external uses like topical applications at the site of envenomation.

4. Conclusion

Irreversible inactivation of snake venom LAAO could be achieved in presence of relatively high concentration of LPG when it appears that the inhibitor binds at multiple sites of the catalytic region. The transfer of a α -hydrogen atom of the amino acid substrate, which activates the functionally inactivate moiety present in such substrate, is essential. An ideal suicidal substrate of LAAO analogous to LPG should bind the catalytic site at low concentration and during catalysis should favor the inactivation reaction far excess over product formation. Finally, low toxicity of the inhibitor is a crucial factor for its safe application.

5. Materials and methods

5.1. Materials

Lyophilized crude venom of *C. adamanteus* and *C. atrox*, FAD, L-Phe, LPG, CHD, DEPC, TNBS, *o*-dianisidine and DEAE-cellulose, α -chymotrypsin were from Sigma, USA. Sephadex G-100 and G-50 were from Amersham Biosciences, Uppsala, Sweden. Horseradish peroxidase (sp. act. 280 U/mg) was from Sisco Research Laboratories Ltd., Mumbai, India. All other reagents were of analytical grade and purchased locally. LPG in powder form was dried to constant weight and weighed gravimetrically to form a working solution of 500 mM in water.

5.2. Purification of LAAO

See Supplementary material.

5.3. Assay of LAAO

The oxidation of L-Phe to the keto acid and H₂O₂ by LAAO was followed by the coupling enzyme horseradish peroxidase (HRP) in presence of the chromophoric dye *o*-dianisidine [46,47]. The colored product formed was followed continuously at 28 °C at $\epsilon_{436nm} = 8310 \text{ M}^{-1} \text{ cm}^{-1}$. The assay mixture contained 70 μ M of L-Phe, 350 mU of HRP, 10 μ M of *o*-dianisidine and the volume was made up to 1-ml by 20 mM Na-phosphate, pH 7.5 (Buffer A). Among hydrophobic

amino acids that act as good substrate of LAAO, L-Phe was selected because the activity of crude venoms used was provided in terms of conversion of L-Phe (0.5 U/mg of protein, Sigma). To confer uniformity, all LAAO assay were done using L-Phe as substrate. One U of LAAO activity is defined as the amount that oxidatively deaminates 1 μ mol of L-Phe/min at 25 °C.

It was reported that LPG acts as a substrate of LAAO and the oxidation product other than H₂O₂ was characterized (3-amino-5-methylene-5H-furan-2-one, $\epsilon_{320\text{nm}} = 13,000 \text{ M}^{-1} \text{ cm}^{-1}$) [26]. For evaluating kinetic parameters, oxidation of LPG was directly measured at 320 nm under similar experimental conditions as stated above where ingredients of coupling assay were excluded. The alternate assay protocol, where formation of H₂O₂ was measured using coupled assay system, was not adapted since with progress of reaction, the product/s interfered with the couple assay system making it less sensitive and erroneous.

5.4. Inactivation of LAAO by LPG

LPG acts both as a substrate and an irreversible inhibitor of LAAO. An aliquot of LAAO (100 μ l, 812.2 nM, 0.812 nmol) in buffer A was mixed with 100 μ l of LPG (final concentration being 5–25 mM) and incubated at 28 °C. Aliquots of 20 μ l were withdrawn at an interval of 5 min and added to 980 μ l of the LAAO assay mixture. The increase in absorbance at 436 nm was monitored for 120 s at 28 °C.

5.5. UV-visible and fluorescence spectra

Enzyme-inhibitor complex was formed as follows: LAAO (0.5 U, 81.22 nM, 0.812 nmoles) and LPG (25 mM) was incubated at 4 °C for 24 h in buffer A. The inactive enzyme was exhaustively dialyzed against buffer A at 4 °C for 24 h to remove excess inhibitor and its oxidation products. UV-visible spectra of the dialysed sample between 200–700 nm were recorded. The dialysed enzyme was concentrated by passing through Microcon centrifugal filter (30 kDa cut off) and subsequently denatured by diluting with 8 M Gdn-HCl in buffer A. The denatured enzyme was dialyzed against the same denaturant for 24 h to remove FAD (modified or unmodified) and the non-covalently bound adduct, if any [30]. The procedure ensures dissociation and removal of FAD and adduct from the enzyme under denatured condition. The absorption spectra of the enzyme inhibitor complex were recorded at 28 °C using an Analytik Jena Specord 200 spectrophotometer. The resulting apoenzyme was finally dialyzed against buffer B to remove the denaturant and UV-visible spectrum was recorded.

To isolate FAD from the LAAO, a portion of the enzyme preparation after denaturation with 8 M Gdn-HCl as described above was passed through the same 30 kDa cut off filter. Fluorescence was recorded using a Hitachi F-7000 spectrofluorimeter.

5.6. Chemical modifications with group-specific reagents

For histidine modification, the enzyme (0.5 U, 81.22 nM, 0.812 nmol) was incubated with 0.5 mM DEPC in buffer A at 22 °C and the residual activity was measured by withdrawing aliquots after 5 min. DEPC was freshly prepared in absolute ethanol. Concentration of DEPC was determined spectrophotometrically by reacting with 10 mM histidine and monitoring the absorbance of *N*-carbethoxyhistidine ($\epsilon_{242\text{nm}} = 3200 \text{ M}^{-1} \text{ cm}^{-1}$) [48]. Reversal of DEPC modification was done by 0.1 M hydroxylamine hydrochloride under identical conditions for 30 min. Arginine modification was performed with 10 mM of freshly prepared CHD (in absolute ethanol) in presence of 50 mM Naborate buffer, pH 8.0 at 22 °C for 5 min [49]. For lysine modification, the enzyme was treated with 10 mM TNBS (from a stock in water) in 100 mM NaHCO₃, pH 8.0 at 30 °C for 5 min, passed through a 1 ml Sephadex G-50 'Spin-column' and the residual activity was measured [50,28]. In all modification experiments, control sets were run where

the same amount of solvent/water was added to the enzyme and the activity was measured.

5.7. Digestion of LPG Inactive LAAO and separation of peptides

Control LAAO and LPG inactivated LAAO free from excess LPG and its catalytic product was digested with α -chymotrypsin at a ratio 50:1(w/w) in 100 mM NH₄HCO₃ buffer pH 8.0 at 37 °C for 18 h. The digested peptide pool was lyophilised and dissolved in HPLC grade water and filtered through 0.22 μ m filter. The peptides were then separated by a Symmetry300 C₁₈ RP-HPLC column (4.6 \times 250 mm). The column was pre-equilibrated with 0.1% (v/v) TFA in water (solvent A). Elution was made by a linear gradient of 70% of solvent D 0.1% (v/v) TFA in acetonitrile over 40 min. Flow rate of 1 ml/min and elution of peptides was followed at 220 and 320 nm and peptides were collected manually and lyophilised for further analysis.

5.8. Mass analysis

The molecular mass of LAAO was determined using an Applied Biosystem 4800 MALDI-TOF/TOF instrument under positive ion modes. The purified enzyme was desalted with a C₄ zip-tip cartridge (Millipore). The salt free protein in 50% acetonitrile containing 0.1% TFA and was applied to the MALDI plate along with the matrix α -cyano-4-hydroxycinnamic acid (CHCA) (12 mg/ml in the same solvent) at a ratio of 1:1 (v/v). The sample was dried at room temperature and mass spectrum was recorded from the accumulation of 1000 single shots. For FAD, the protein was denatured, precipitated with methanol/acetonitrile (1:1), vortexed and centrifuged to get a clear supernatant. The supernatant was dried, reconstituted with CHCA solution and applied to the MALDI plate for mass determination using the negative ion detection mode. Protease digested peptides after RP-HPLC was directly spotted on MALDI plate along with CHCA for mass analysis.

5.9. Sequence analysis and modeling study

Amino acid sequence of LAAO from *C. adamanteus* (Eastern Diamondback rattle snake) and *C. atrox* (Western Diamondback rattle snake) were obtained from UniprotKB/Swiss-prot database (<http://www.uniprot.org>) (protein database O93364 and P56742, respectively). The sequences were aligned with that of other LAAOs such as *Agkistrodon halys* (PDB ID: 1REO), *Vipera ammodytes ammodytes* (PDB ID: 3KVE) and *C. rhodostoma* (PDB ID: 2IID) using 'Clustal W' for homology determination (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). The protein structure of LAAO from *C. adamanteus* was predicted from homology modeling using two different servers, 'Swiss model' (<http://www.swissmodel.expasy.org>) [51] and 'CPH model' (<http://www.cbs.dtu.dk/services/CPHmodels>) [52] using *Agkistrodon halys* (PDB ID: 1REO) as the reference structure. Visualization and preparation of illustrations were done by PyMol software [32].

Financial support

JM was supported by CSIR-NET fellowship. Research was supported by the institutional facilities.

Acknowledgements

Mr. Manidip Shasmal and Biprashekhkar Chakraborty of this institute helped in modeling studies.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2013.01.010.

References

- [1] Curti B., Ronchi S., Simonetta M.P. (1992) D- and L-amino acid oxidases. In: F. Muller (Ed.), In Chemistry and Biochemistry of Flavoenzymes Boca Raton, FL: CRC Press; vol. 3, pp. 69–94.
- [2] Du X.Y., Clemetson K.J. (2002) Snake venom L-amino acid oxidases. *Toxicol.* 40, 659–665.
- [3] Mandal S., Bhattacharyya D. (2008) Two L-amino acid oxidase isoenzymes from Russell's viper (*Daboia russelli russelli*) venom with different mechanism of inhibition by substrate analogs. *FEBS J.* 275, 2078–2095.
- [4] Fitzpatrick P.F. (2004) Carbanion versus hydride transfer mechanisms in flavoprotein-catalyzed dehydrogenations. *Bioorg. Chem.* 32, 125–139.
- [5] Sakurai Y., Takatsuka H., Yoshioka A., Matsui T., Suzuki M., Titani K. et al. (2001) Inhibition of human platelet aggregation by L-amino acid oxidase purified from *Naja naja kaouthia* venom. *Toxicol.* 39, 1827–1833.
- [6] Lu Q.M., Wei Q., Jin Y., Wei J.F., Wang W.Y., Xiong Y.L. (2002) L-Amino acid oxidase from *Trimeresurus jerdoni* snake venom: purification, characterization, platelet aggregation-inducing and antibacterial effects. *J. Nat. Toxins.* 11, 345–352.
- [7] Suhr S.M., Kim D.S. (1996) Identification of snake venom substrate that induces apoptosis. *Biochem. Biophys. Res. Commun.* 224, 134–139.
- [8] Suhr S.M., Kim D.S. (1999) Comparison of the apoptotic pathways induced by L-amino acid oxidase and hydrogen peroxidase. *J. Biochem.* 125, 305–309.
- [9] Torii S., Yamane K., Mashima T., Haga N., Yamamoto K., Fox J.W. et al. (2000) Molecular cloning and functional analysis of apoxin I, a snake venom-derived apoptosis-inducing factor with L-amino acid oxidase activity. *Biochemistry.* 39, 3197–3205.
- [10] Yang C.A., Cheng C.H., Liu S.Y., Lo C.T., Lee J.W., Peng K.C. (2011) Identification of antibacterial mechanism of L-amino acid oxidase derived from *Trichoderma harzianum* ETS 323. *FEBS J.* 278, 3381–3394.
- [11] Chu C.C., Paul W.E. (1997) Fig 1, an interleukin 4-induced mouse B cell gene isolation by cDNA representational difference analysis. *Proc. Natl Acad. Sci. U.S.A.* 94, 2507–2512.
- [12] Pawelek P.D., Cheah J., Coulombe R., Macheroux P., Ghisla S., Vrieling A. (2000) The structure of L-amino acid oxidase reveals the substrate trajectory into an enantiomerically conserved active site. *EMBO J.* 19, 4204–4215.
- [13] Moustafa I.M., Foster S., Lyubimov A.Y., Vrieling A. (2006) Crystal structure of LAAO from *Calloselasma rhodostoma* with an L-phenylalanine substrate: insight into structure and mechanism. *J. Mol. Biol.* 15, 991–1002.
- [14] Franca S.C., Kashima S., Roberto P.G., Marins M., Tielci F.K., Pereira J.O. et al. (2007) Molecular approaches for structural characterization of Bothrops L-amino acid oxidases with anti protozoal activity: cDNA cloning, comparative sequence analysis, and molecular modeling. *Biochem. Biophys. Res. Commun.* 355, 302–306.
- [15] Walse C. (1982) Suicide substrate: mechanism-based enzyme inactivator. *Tetrahedron.* 38, 871–909.
- [16] Ghisla S., Wenz A., Thrope C. (1980) Suicide substrates as irreversible inhibitors of flavoenzymes. In: U Brodbeck (Ed.), In Enzyme Inhibitors. Weinheim, Germany: Gmbh, pp. 43–60.
- [17] Cummings J.G., Thrope C. (1994) 3-Methylene-octanoyl-CoA and 3-methyl-trans-2-octenoyl-CoA: two new mechanism-based inhibitors of medium chain acyl-CoA dehydrogenase from pig kidney. *Biochemistry.* 33, 788–797.
- [18] Fendrich G., Abeles R.H. (1982) Mechanism of action of butyryl-CoA dehydrogenase: reactions with acetylenic, olefinic, and fluorinated substrate. *Biochemistry.* 21, 6685–6695.
- [19] Powell P.J., Thrope C. (1988) 2-Octenoyl coenzyme A is a mechanism-based inhibitor of pig kidney medium-chain acyl coenzyme A dehydrogenase: isolation of the target peptide. *Biochemistry.* 27, 8022–8028.
- [20] Freund K., Mizzer J.P., Dick W., Thrope C. (1985) Inactivation of general acyl-CoA dehydrogenase from pig kidney by 2-alkynol coenzyme A derivatives: initial aspects. *Biochemistry.* 25, 5996–6002.
- [21] Lundberg N.N., Thrope C. (1993) Inactivation of short-chain acyl-coenzyme A dehydrogenase from pig liver by 2-pentynoyl-coenzyme A. *Arch. Biochem. Biophys.* 306, 454–459.
- [22] Frerman F.E., Miziorko H.M., Beckmann J.D. (1980) Enzyme-activated inhibitors, alternate substrates, and a dead end inhibitor of general acyl-CoA dehydrogenase. *J. Biol. Chem.* 255, 11192–11198.
- [23] Gomes B., Fendrich G., Abeles R.H. (1981) Mechanism of action of glutaryl-CoA and butyryl-CoA dehydrogenases. purification of glutaryl-CoA dehydrogenase. *Biochemistry.* 20, 1481–1490.
- [24] Silverman R.B. (1995) Mechanism-based enzyme inactivators. *Methods Enzymol.* 249, 240–283.
- [25] Marcotte P., Walsh C. (1976) Vinylglycine and propargylglycine: complementary suicide substrates for L-amino acid oxidase and D-amino acid oxidase. *Biochemistry.* 15, 3070–3076.
- [26] Marcotte P., Walsh C. (1978) Sequence of reactions which follows enzymatic oxidation of propargylglycine. *Biochemistry.* 17, 5613–5619.
- [27] Marcotte P., Walsh C. (1978) Properties of D-amino acid oxidase covalently modified upon its oxidation of D-propargylglycine. *Biochemistry.* 17, 2864–2868.
- [28] Nath S., Brahma A., Bhattacharyya D. (2003) Extended application of gel-permeation chromatography by spin column. *Anal. Biochem.* 320, 199–206.
- [29] Kitz R., Wilson I.B. (1962) Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol. Chem.* 237, 3245–3249.
- [30] Rothman S.C., Johnston J.B., Lee S., Walker J.R., Poulter C.D. (2008) Type II isopen-tenyl diphosphate isomerase: irreversible inactivation by covalent modification of flavin. *J. Am. Chem. Soc.* 130, 4906–4913.
- [31] Massey V., Curti B. (1967) On the reaction mechanism of *Crotalus adamanteus* L-amino acid oxidase. *J. Biol. Chem.* 242, 1259–1267.
- [32] The PyMOL Molecular Graphics System, Version 1.5.0.4. Schrödinger, LLC
- [33] Maycock A.L. (1980) Flavin suicide inhibitor adducts. *Methods Enzymol.* 66, 294–302.
- [34] Binda C., Hubalek F., Li M., Herzir Y., Sterling J., Edmondson D.E. et al. (2004) Crystal structure of monoamine oxidase B in complex with four inhibitors of the N-propargylaminoindan class. *J. Med. Chem.* 47, 1767–1774.
- [35] Binda C., Hubalek F., Li M., Herzir Y., Sterling J., Edmondson D.E. et al. (2005) Binding of rasagiline-related inhibitors to human monoamine oxidase: a kinetic and crystallographic analysis. *J. Med. Chem.* 48, 8148–8154.
- [36] Yang M., Culhane J.C., Szweczek L.M., Cole P.A., Yu H. (2007) Structural basis of histone demethylation by LSD1 revealed by suicide inactivation. *Nat. Struct. Mol. Biol.* 14, 535–539.
- [37] Chen Z.W., Zhao G., Martinovic S., Jorns M.S., Mathews F.S. (2005) Structure of the sodium borohydride-reduced N-(cyclopropyl) glycine adduct of the flavoenzyme monomeric sarcosine oxidase. *Biochemistry.* 44, 15444–15450.
- [38] Høj P.B., Condron R., Traeger J.C., McAuliffe J.C., Stone B.A. (1992) Identification of glutamic acid 105 at the active site of *Bacillus amyloliquefaciens* 1,3–1,4-β-D-glucan 4-glucanohydrolase using epoxide-based inhibitors. *J. Biol. Chem.* 267, 25059–25066.
- [39] Mueller M.J., Andberg M., Haeggström J.Z. (1998) Analysis of the molecular mechanism of substrate-mediated inactivation of Leukotriene A₄ Hydrolase. *J. Biol. Chem.* 273, 11570–11575.
- [40] Hess D.T., Matsumoto A., Kim S.O., Marshall H.E., Stamler J.S. (2005) Protein S-nitrosylation: purview and parameters. *Nat. Rev.* 6, 150–166.
- [41] Drawz S.M., Bonomo R.A. (2010) Three decades of β-lactamase inhibitors. *Clin. Microbiol. Rev.* 23, 160–201.
- [42] Zhang H., Lin H.L., Kenaan C., Hollenberg P.F. (2011) Targeting of the highly conserved threonine 302 residue of cytochromes P450 2B family during mechanism-based inactivation by aryl acetylenes. *Arch. Biochem. Biophys.* 507, 135–143.
- [43] Fraaije M.W., Mattevi A. (2000) Flavoenzymes: diverse catalysts with recurrent features. *Trends Biochem. Sci.* 25, 126–132.
- [44] Stein W.H., Moore S. (1954) The free amino acids of human blood plasma. *J. Biol. Chem.* 211, 915–926.
- [45] Nasset E.S., Heald F.P., Calloway D.H., Margen S., Schneeman P. (1979) Amino acids in human blood plasma after single meals of meat, oil, sucrose, and whiskey. *J. Nutr.* 109, 621–630.
- [46] Ponnudurai G., Chung M.C., Tan N.H. (1994) Purification and properties of the L-amino acid oxidase from Malayan pit viper (*Calloselasma rhodostoma*) venom. *Arch. Biochem. Biophys.* 313, 373–378.
- [47] Wei X.L., Wei J.F., Li T., Qiao L.Y., Liu Y.L., Huang T. et al. (2007) Purification, characterization and potent lung lesion activity of an L-amino acid oxidase from *Agkistrodon blomhoffii ussuriensis* snake venom. *Toxicol.* 50, 1126–1139.
- [48] Ovadi J., Libor S., Elodi P. (1967) Spectrophotometric determination of histidine in protein with pyrocarbonate. *Biochim. Biophys. Acta.* 2, 455–458.
- [49] Cheng K.C., Nowak T. (1989) Arginine residues at the active site of avian liver phosphoenolpyruvate carboxylase. *J. Biol. Chem.* 264, 3317–3324.
- [50] Hondal R.J., Ma S., Caprioli R.M., Hill K.E., Burk R.F. (2001) Heparin-binding histidine and lysine residue of rat selenoprotein P. *J. Biol. Chem.* 276, 15823–15831.
- [51] Arnold K., Bordoli L., Kopp J., Schwede T. (2006) The SWISS-MODEL workspace: a web-based environment protein structure homology modeling. *Bioinformatics.* 22, 195–201.
- [52] Nielsen M., Lundegaard C., Lund O., Petersen T.N. CPHmodels-3.0-Remote homology modeling using structure guided sequence profiles *Nucleic Acids Research* 2010;38: 576–581.