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L-Amino-acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*

Comparative sequence analysis and charaterization of active and inactive forms of the enzyme

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Here we report the cDNA-deduced amino-acid sequence of L-amino-acid oxidase (LAAO) from the Malayan pit viper *Calloselasma rhodostoma*, which shows 83% identity to LAAOs from the Eastern and Western diamondback rattlesnake (*Crotalus adamanteus* and *Crotalus atrox*, respectively). Phylogenetic comparison of the FAD-dependent ophidian LAAOs to FAD-dependent oxidases such as monoamine oxidases, D-amino-acid oxidases and tryptophan 2-monooxygenases reveals only distant relationships. Nevertheless, all LAAOs share a highly conserved dinucleotide-binding fold with monoamine oxidases, tryptophan 2-monooxygenases and various other proteins that also may have a requirement for FAD. In order to characterize *Ca. rhodostoma* LAAO biochemically, the enzyme was purified from snake venom to apparent homogeneity. It was found that the enzyme undergoes inactivation by either freezing or increasing the pH to above neutrality. Both inactivation processes are fully reversible and are associated with changes in the UV/visible range of the flavin absorbance spectrum. In addition, the spectral characteristics of the freeze-and pH-induced inactivated enzyme are the same, indicating that the flavin environments are similar in the two inactive conformational forms. Monovalent anions, such as Cl^-, prevent pH-induced inactivation. LAAO exhibits typical flavoprotein oxidase properties, such as thermodynamic stabilization of the red flavin semiquinone radical and formation of a sulfite adduct. The latter complex as well as the complex with the competitive substrate inhibitor, anthranilate, were only formed with the active form of the enzyme indicating diminished accessibility of the flavin binding site in the inactive form(s) of the enzyme.

Keywords: L-amino-acid oxidase; flavoprotein; amino-acid sequence; phylogeny; reversible inactivation.

L-Amino-acid oxidase (LAAO) occurs in many different organisms such as bacteria (*Corynebacterium* [1], and *Proteus* [2]), cyanobacteria (*Synechococcus* [3]), fungi (*Neurospora crassa* [4]), green algae (*Chlamydomonas rheinhardtii* [5]), and, most prominently, venomous snakes (such as crotalids, elapids and viperids [6]). LAAO is the only FAD-dependent oxidase found in snake venom and is thought to contribute to its toxicity, possibly through generation of hydrogen peroxide formed as a result of reoxidation of the transiently reduced flavin cofactor by molecular oxygen (Scheme 1). However, the exact role of this enzyme in snake venom is not yet understood [6]. LAAOs from bacterial, fungal and plant sources appear to be involved in the utilization of ammonia as a nitrogen source. LAAOs from different sources are also distinct with regard to molecular mass, substrate specificity, post-translational modifications (glycosylation) and their regulation. This diversity suggests that LAAOs have undergone large evolutionary changes since their separation from a putative ancestral protein.

As LAAO from snake venom, in particular that of *Crotalus adamanteus* (eastern diamondback rattlesnake) can be rapidly and easily purified [7], it has become an attractive subject of enzymological, kinetic, and mechanistic investigations (reviewed in [8]). The peculiar and fully reversible freeze- and pH-induced inactivation of the enzyme was the subject of detailed reports in the 1960s and seventies [7,9–11]. It can be summarized as follows: (a) inactivation occurs upon freezing of the enzyme to subzero temperatures (with a maximal effect at −20 °C) with the rate of inactivation depending on pH and buffer composition; (b) pH-induced inactivation takes place upon increasing the pH to above neutrality in the absence of monovalent anions such as chloride, which prevent this type of inactivation. The most favourable reactivation conditions for both inactivated forms involve heat treatment at low pH

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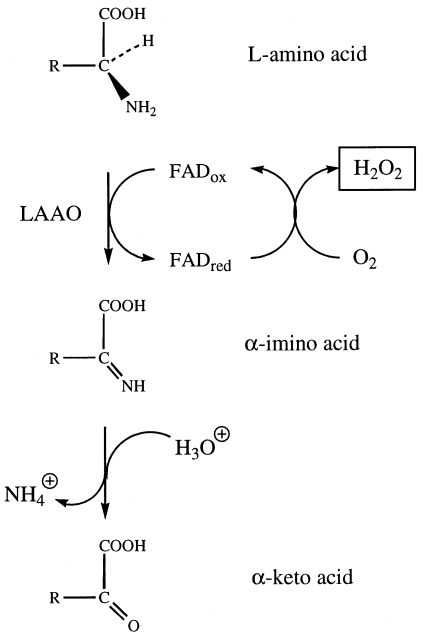
Abbreviation: LAAO, L-amino-acid oxidase.
Enzyme: L-amino acid oxidase (EC 1.4.3.2).



Scheme 1.

(e.g. 37 °C and pH 5 [9,10]). The activation/inactivation process is associated with shifts of the absorbance maxima of the FAD cofactor, which can be utilized to monitor the interconversions. Because the changes in the UV/visible absorbance and circular dichroism spectrum appear to be different for the freeze- and pH-inactivated form, it was suggested that these two inactive forms are structurally distinct [10]. Although the enzyme from *Cr. adamanteus* was already crystallised in 1960 by Wellner [7], the first crystal structure of a LAAO was reported only recently [12]. This structure was obtained with the enzyme obtained from the venom of the Malayan pit viper *Calloselasma rhodostoma*. Unfortunately, very little biochemical and kinetic data is currently available for the enzyme, and, moreover, some of the studies with this LAAO have produced a number of contrasting results concerning the nature of the cofactor (FMN instead of FAD) and the absence of a reversible inactivation of the enzyme as described above [13]. In view of this lack of information, we carried out a basic biochemical and enzymatic characterization of LAAO from *Ca. rhodostoma* in order to provide a sound basis for a comparison with the wealth of data available for LAAO from the American species *Cr. adamanteus* and *Cr. atrox*.

EXPERIMENTAL PROCEDURES

Chemicals and enzymes

All reagents and chemicals were from Fluka, Buchs, Switzerland. Horseradish peroxidase (EC 1.11.1.7) was from Merck, Darmstadt, Germany. LAAO was obtained from the venom of the Malayan pit viper (*Ca. rhodostoma*). The LAAO containing side fractions from purification of ancrod (a thrombine-like serine protease; Knoll AG/BASF AG, Ludwigshafen, Germany) were purified further by gel filtration with a Superdex 200 HiLoad column (column size 26/60). The core fractions from the eluate (approx. 50% of the total eluted LAAO) were collected, and analyzed by SDS/PAGE [14] and UV/visible spectroscopy. Fractions with a ratio $A_{280}/A_{450} < 9.7$ were pooled and concentrated by ultrafiltration through Microcon concentrators (Millipore). Fractions containing protein impurities were purified further by the same method. All operations were carried out in 100 mM Tris/phosphate buffer, pH 8.

L-Amino-acid oxidase assay

Activity of LAAO was determined in 0.1 M Tris/phosphate buffer, pH 7.8 and at 25 °C using an enzyme-coupled assay. In this assay, hydrogen peroxide generated by LAAO was used by horseradish peroxidase to oxidize *o*-dianisidine to the radical cation which was spectrophotometrically monitored at $\lambda = 440$ nm; 1-mL assay mixtures contained 10 μL horseradish peroxidase (1 mg·mL^{-1}), 50 μL of an *o*-dianisidine solution (8 mM, 20% Triton X-100) and 10 μL of a 0.1 M L-leucine solution. The reaction was started by addition of 5–10 μL of the LAAO-solution.

Cloning and sequencing of the cDNA of L-amino-acid oxidase

All molecular methods were adapted from those reported previously [15,16]. A Uni ZAPII cDNA library obtained with a poly(A)$^+$ RNA preparation of *Ca. rhodostoma* venom glands was used to screen for the cDNA of LAAO [17]. A N-terminal amino-acid sequence comprising 40 amino acids of LAAO was determined by Edman sequencing with a Hewlett Packard Protein Sequencer (model HP G1000A) using HP PCS G1017A sequencing columns suitable for poly(vinylidene difluoride) membrane bound proteins. The standard method provided by the manufacturer (N-terminal 4.0) was used. An internal amino-acid sequence of *Ca. rhodostoma* LAAO was obtained from a cyanogen bromide cleavage product, isolated on SDS/PAGE, electroblotted to a poly(vinylidene difluoride) membrane and subjected to Edman sequencing as described above. Highly conserved amino-acid regions in each of the amino-acid sequences available were selected for construction of oligonucleotide primers. The wobble bases in these regions were determined according to the usage in the *Cr. adamanteus* cDNA [18]. These primers were used to probe the *Ca. rhodostoma* cDNA bank for the presence of the LAAO-cDNA. A PCR product of ≈ 300 bp was obtained indicating that the desired LAAO-cDNA is present in the cDNA bank. Plasmids containing the LAAO-cDNA were identified by inverse PCR and inserts of approximately 2.6 kb, encoding for *Ca. rhodostoma* LAAO, were sequenced by generating progressive unidirectional deletions with the Erase-a-base system (Promega).

Determination of the extinction coefficient of FAD

FAD bound to *Ca. rhodostoma* LAAO was released by addition of SDS (final concentration 0.1% v/v). UV/visible

Table 1. Comparison of enzyme activities of the active and inactive forms of *Ca. rhodostoma* L-amino-acid oxidase (in %).

	Heat treatment (10 min, 37 °C)	
LAAO	Before	After
Active form[a]	100	84.5
pH-inactivation[b]	85	8.5
Freeze-inactivation[c]	0.75	0.6

[a] Active enzyme in 0.2 M sodium acetate, pH 5 (25 μL) diluted into 975 μL, 0.1 M Tris/phosphate buffer, pH 7.8 containing 10 mM sodium chloride; sample was handled at 20 °C before heat treatment; [b] conditions as above but buffer lacked NaCl; [c] enzyme stored at −20 °C for at least 24 hours in 0.1 M Tris/phosphate buffer, pH 7.8.

spectra were recorded before and after addition of SDS and the extinction coefficient of FAD bound to LAAO was calculated using an ε_{max} at 450 nm of 11 300 $M^{-1} \cdot cm^{-1}$ for free FAD. This method yields an ε_{max} at 459 nm (absorbance maximum of freeze-inactived LAAO) of 11600 $M^{-1} \cdot cm^{-1}$. All UV/visible absorbance spectra were recorded with a thermostatted Uvikon 933 (Kontron Instruments).

RESULTS AND DISCUSSION

Phylogenetic relationship to other FAD-dependent oxidases and emergence of a highly conserved FAD-binding motif

The open reading frame of LAAO from *Ca. rhodostoma* comprises 516 amino acids (accession no. AJ271725). This amino-acid sequence was used for a phylogenetic analysis. As expected, the three known sequences of ophidian LAAOs form a family of proteins (ophidian LAAOs, see Fig. 1). The mere 83% sequence identity found between *Ca. rhodostoma* LAAO and the LAAO from the two *Crotalus* species reflects the geographic separation of Asian and American pit vipers some 20 million years ago and subsequent speciation. These ophidian LAAOs are more distantly related to the interleukin 4-induced Fig. 1 protein from *Mus musculus* [18], a putative LAAO from *Bacillus subtilis*, and a protein of unknown function from *Bacillus cereus* (see Fig. 1). However, there is no apparent relationship to LAAOs from any other organism including *Neurospora crassa*, *Chlamydomonas rheinhardtii* and *Synechococcus*. This result from the phylogenetic reconstruction is in agreement with the biochemical diversity of LAAOs isolated and described from various sources as mentioned in the introduction, and suggests that divergence from a putative ancestral protein occurred very early in the

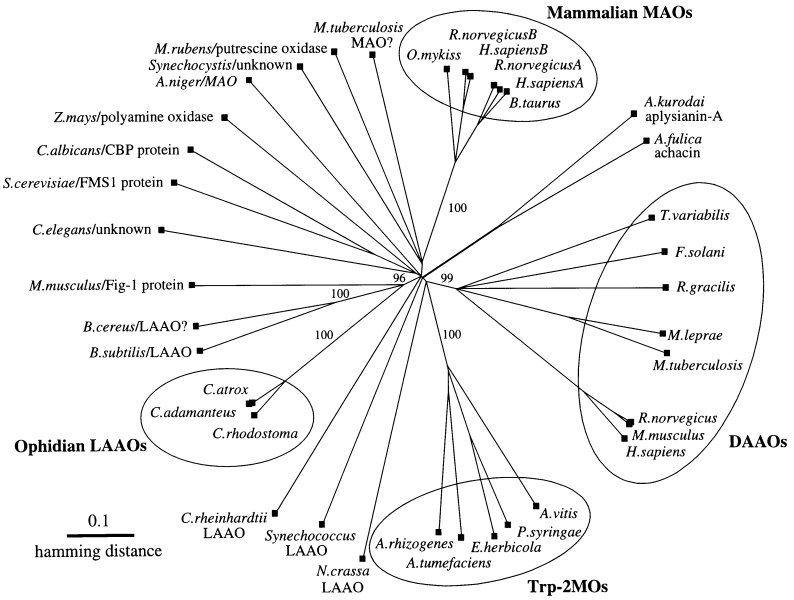


Fig. 1. Unrooted phylogenetic tree. Phylogenetic reconstruction was carried out with the program SPLITSTREE2.4 by D. Huson based on the split decomposition method described recently [28]. The complete amino-acid sequences of the proteins were used for analysis. The circles indicate the suggested families of proteins. Proteins from the following species were used in construction of the unrooted phylogenetic tree: LAAO from *Bacillus cereus*, *Bacillus subtilis*, *Ca. rhodostoma*, *Chlamydomonas reinhardtii*, *Cr. adamanteus*, *Cr. atrox*, *Neurospora crassa, and Synechococcus*; interleukin 4-induced Fig-1 protein from *Mus musculus*; monoamine oxidase from *Aspergillus niger*, *Bos taurus*, *Homo sapiens*A, *Homo sapiens*B, *Oncorhynchus mykiss, Rattus norvegicus*A, *Rattus norvegicus*B and *Mycobacterium tuberculosis* (putative monoamine oxidase); tryptophan 2-monoxygenase from *Agrobacterium rhizogenes*, *Agrobacterium tumefaciens, Agrobacterium vitis*, *Erwinia herbicola* and *Pseudomonas syringae*; putrescine oxidase from *Micrococcus rubens*; polyamine oxidase from *Zea mays*; protein of unknown function from *Synechocystis* (D64000); achacin from *Achatina fulica*; aplysianin-A from *Aplysia kurodai*; corticosteroid-binding protein (CBP1) from *Candida albicans*; steroid-binding protein FMS1 from *Saccharomyces cerevisiae*; protein of unknown function from *Caenorhabditis elegans*; D-amino-acid oxidase from *Fusarium solani, Homo sapiens*, *Mus musculus, Mycobacterium leprae, Mycobacterium tuberculosis*, *Rattus norvegicus*, *Rhodotorula gracilis* and *Trigonopsis variabilis*.

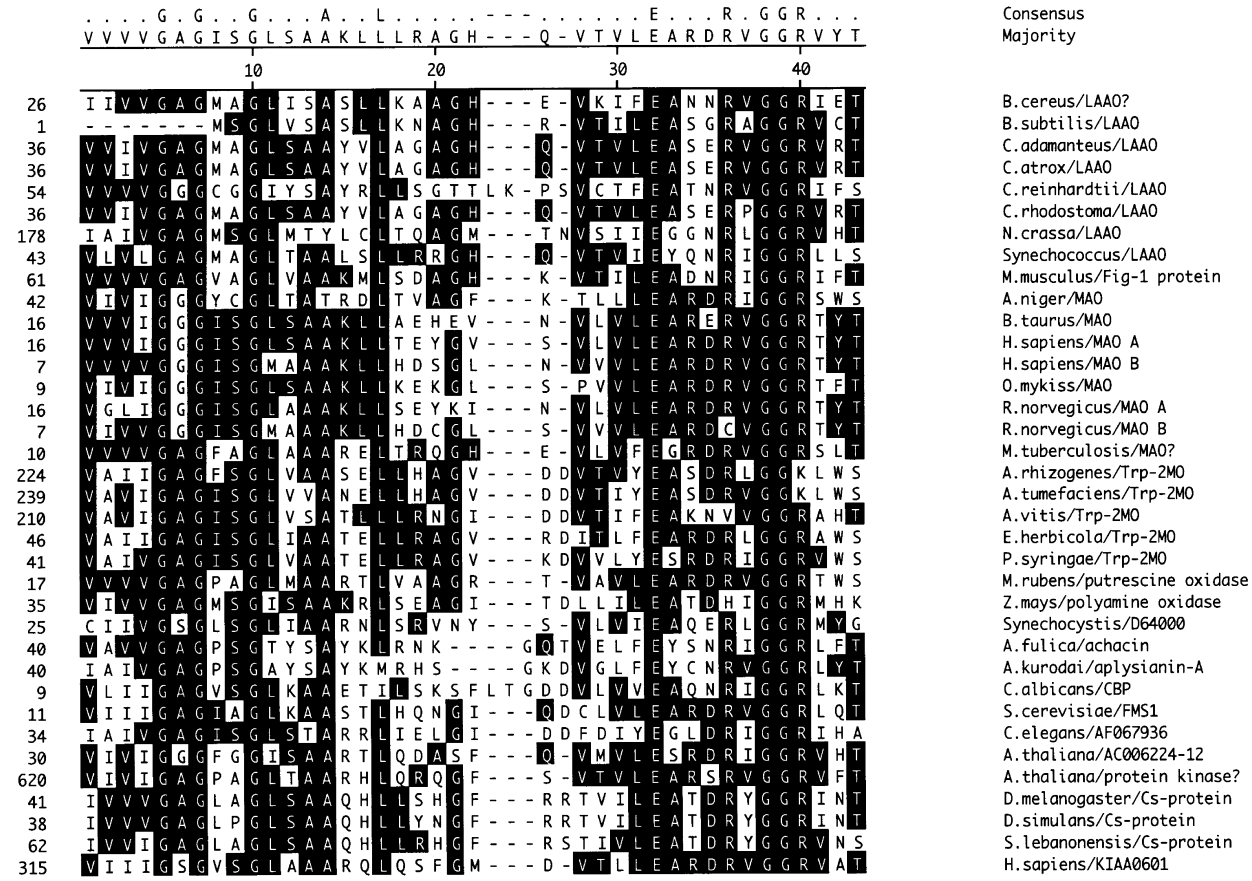


Fig. 2. Multiple sequence alignment of an extended dinucleotide-binding fold in L-amino-acid oxidases (LAAO), (mono-)amine oxidases (MO), tryptophan 2-monooxygenases (Trp-2MO) and other putative FAD-containing proteins. Consensus and majority sequences are shown on top. The numbering on the left indicates the position of the first amino acid shown in the protein sequence. Species names and function (if known) of the protein are given on the right. The alignment was performed using CLUSTALX .

evolution of LAAOs. Also included in the unrooted phylogenetic tree (Fig. 1) are other FAD-dependent enzymes such as D-amino-acid oxidases, monoamine oxidases and tryptophan 2-monoxygenases. Each of these protein families shows different degrees of divergence, but none of them has a close relationships with LAAOs. Despite this divergence on a whole sequence level, the FAD-binding fold shows a very high degree of conservation as shown in Fig. 2. The main feature of this fold is a highly conserved R-h-G-G-R-h-x-(T or S) motif extending beyond the dinucleotide-binding fold described by Wierenga [19]. This conserved region is found in all LAAOs, monoamine oxidases, tryptophan 2-monoxygenases, in a putrescine and polyamine oxidase, as well as a number of other less well characterized proteins (see Figs 1 and 2). These proteins appear to form a subfamily in the FAD-containing superfamily described recently [20]. Therefore, we suggest that the presence of this motif is indicative of a FAD-binding site. It is, however, not required for FAD binding as, for example, D-amino-acid oxidases lack this conserved region. In this context it is also worth noting that the second conserved arginine and the following, highly conserved, threonine (see Fig. 2) were found to be essential for covalent incorporation of FAD in human monoamine oxidase B [21].

Properties of the active and inactive form of *Ca. rhodostoma* LAAO

Activity of purified LAAO was determined using the horseradish peroxidase coupled assay as described under Experimental procedures. A k_{cat} value of 19.4 s^{-1} was determined with L-leucine as substrate (K_m = 0.56 mM) in 0.1 M Tris/Cl, 10 mM NaCl at pH 7.8 and at 25 °C. These values compare to k_{cat} = 3.3 s^{-1} and K_m = 0.63 mM measured with the same method by Ponnudurai *et al*. [13]. When purified LAAO was frozen at −20 °C in 0.1 M Tris/phosphate buffer, pH 7.8, less than 1% of the original activity was recovered upon thawing (Table 1, compare first and third row). Heat treatment (37 °C, 10 min) of the freeze inactivated form of the enzyme in this buffer did not restore enzyme activity (Table 1, compare activities in the third row). However, complete reactivation was achieved by placing the enzyme in 0.2 M sodium acetate buffer, pH 5, followed by heat treatment. This reactivation process is accompanied by a bathochromic shift of the visible absorbance band from 459 to 465 nm in the UV/visible spectrum of the flavin as shown in Fig. 3. The spectral changes are similar to those reported for the *Cr. adamanteus* enzyme [9,10]. Inactivation of active LAAO also occurs when the pH is raised from 5 to 7.8 followed by heat

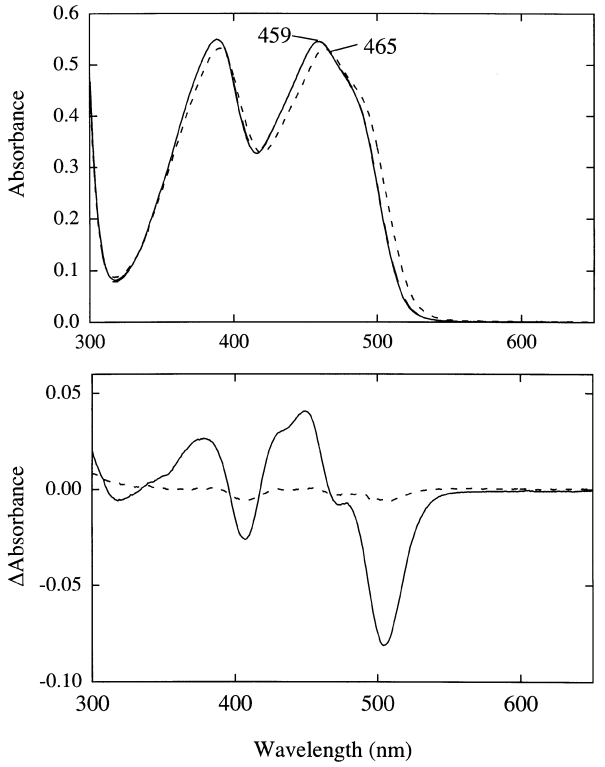


Fig. 3. Comparison of the UV-visible-absorbance spectrum of freeze- and pH-inactivated with active *Ca. rhodostoma* LAAO. LAAO in 0.1 M Tris/phosphate buffer, pH 7.8. The absorbance spectrum of the freeze-inactivated LAAO is represented by the solid line (λ_{max} at 459 nm). The spectrum of the pH-inactivated LAAO is very similar to that of the freeze-inactivated LAAO (see dashed line in the difference spectrum in the lower panel). Both spectra were recorded in 0.1 M Tris/phosphate buffer, pH 7.8. The spectrum of active LAAO (in 0.1 M Tris/phosphate, containing 10 mM sodium chloride, pH 7.8) is represented by the dashed line in the top panel λ_{max} at 465 nm). The difference spectrum of the freeze-inactivated form minus the active form is shown as a solid line in the lower panel.

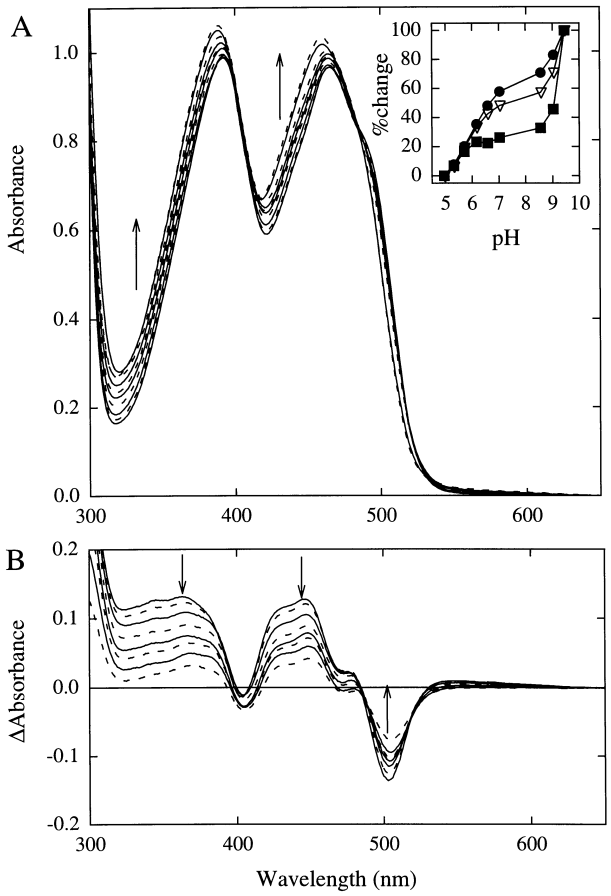


Fig. 4. pH-titration of active *Ca. rhodostoma* LAAO. LAAO in 0.2 M sodium acetate at 25 °C. The pH of the sample was raised by addition of solid sodium carbonate. Spectra shown in (A) were recorded at the following pH values: 4.95, 5.31, 5.71, 6.16, 6.58, 7.03, 8.56, 9.04, and 9.45 (changes occurred in the direction of the arrows). Insert: the percentage change at the following wavelength: (●) 350 nm, (▽) 441 nm and (■) 501 nm as a function of pH. (B) The difference spectra between the last spectrum at pH 9.45 and the spectra at lower pH values.

treatment (Table 1, compare activities in the second row). This type of inactivation is largely prevented by the presence of 10 mM NaCl (Table 1, compare activities in the first row). Again, this pH inactivation and its prevention by chloride was also described for the *Cr. adamanteus* enzyme [10]. However, Coles *et al*. have reported spectral differences between the freeze-inactivated and pH-induced inactivated form of LAAO from *Cr. adamanteus* [10]. In the case of *Ca. rhodostoma* LAAO, the observed spectral differences between the freeze and pH-induced inactivated enzyme (Fig. 3, lower panel) are very small indicating that the flavin experiences very similar, if not identical environments in the freeze- and pH-induced inactive state.

The pH-inactivation of LAAO was further characterized by pH-titration of active LAAO in 0.2 M sodium acetate buffer, pH 5 (Fig. 4). Raising the pH induces a hypsochromic shift of the flavin absorbance as depicted in Fig. 4A. Importantly, these spectral changes occur without loss of enzymatic activity. Above pH 9 a further, pronounced spectral change occurs mainly between 500 and 520 nm, which is accompanied by a ≈75% loss of activity over a period of 10 min at pH 9.45. The biphasic character of the spectral changes is clear from a plot of the percentage of the total changes as a function of pH, as shown in the inset of Fig. 4A. Hence, it can be concluded that although the spectral changes are similar throughout the pH titration, spontaneous inactivation does not occur (at this temperature) until pH 9. In contrast to the protective effect of chloride at lower pH (7.8), this pH driven inactivation is not prevented in the presence of 10 mM Cl^-.

Studies with *Cr. adamanteus* LAAO have also demonstrated that inactivation of the enzyme affect typical flavoprotein oxidase properties such as the formation of the red anionic semiquinone and the N(5)-sulfite adduct [9,10]. For the sake of comparison, these properties were also investigated using the freeze-inactivated and activated form of the *Ca. rhodostoma* enzyme.

Anaerobic photoreduction

As shown in Fig. 5, anaerobic photoreduction of *Ca. rhodostoma* LAAO gives rise to spectral changes

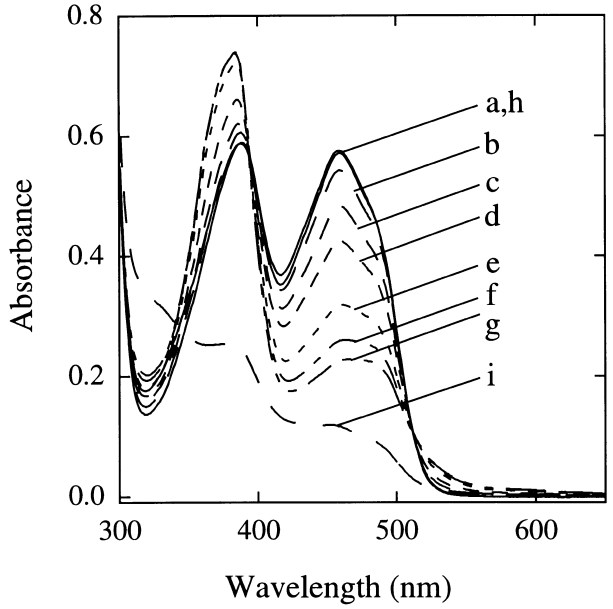


Fig. 5. Anaerobic photoreduction of *Ca. rhodostoma* L-amino-acid oxidase. Forty-eight micromolar LAAO in 0.1 M Tris/Cl buffer, pH 7.8 containing 1 mM EDTA and 1 μM methyl viologen (paraquat) was made anaerobic by repeated cycles of evacuation and flushing with argon (1 p.p.m. oxygen). The first spectrum shown was recorded after anaerobic conditions were established (a). The sample was then photoreduced with light using a conventional slide projector as a light source. Spectra shown were recorded after 5 min (b), 25 min (c), 45 min (d), 90 min (e), 135 min (f), and 170 min (g), respectively. The fully photoreduced FAD is represented by spectrum (i). The cuvette was then opened to air and the spectrum of the reoxidised sample was recorded (h).

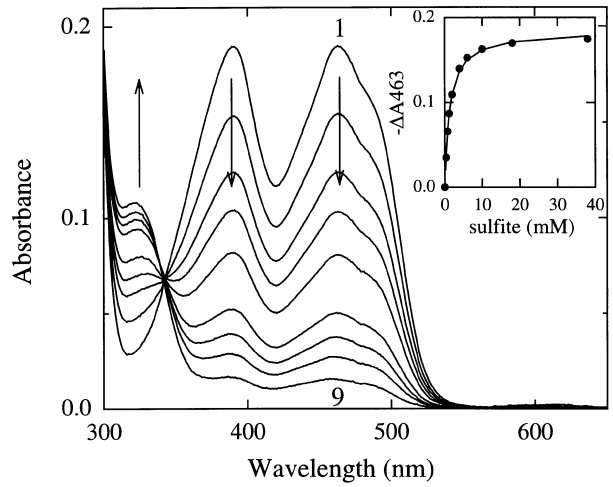


Fig. 6. Titration of activated *Ca. rhodostoma* LAAO with sodium sulfite. Sodium sulfite in 0.1 M Tris buffer, 10 mM sodium chloride, pH 7.8. The spectra shown (from top to bottom) were recorded at final sulfite concentrations of 0, 0.4, 0.8, 1.2, 2, 4, 6, 10 and 38 mM. The inset shows the absorbance difference at 463 nm (●) and a hyperbolic fit to the data [f(x) = $A(463)_{max}$ × [sulfite]/K_d x [sulfite]] yielding a dissociation constant (K_d = 2 mM) for the formation of the FAD–sulfite complex.

indicative for the formation of a red flavin semiquinone. This flavin radical species has an extinction coefficient of 15 460 $M^{-1} \cdot cm^{-1}$ at λ_{max} = 384 nm. Its extent of formation and its UV/visible spectrum is quite similar to that reported for the *Cr. adamanteus* enzyme [22]. However, photoreduction of the activated form of *Ca. rhodostoma* LAAO is approximately twofold faster than that of the freeze-inactivated form. Also, further reduction to the fully reduced FAD form is faster with activated *Ca. rhodostoma* LAAO as compared to the freeze-inactivated form.

Reaction with sulfite, formation of the N(5)-sulfite covalent adduct

A difference of more than two orders of magnitude was found in the affinity to sulfite of the freeze-inactivated ($K_d \approx 0.25$ M) vs. activated *Ca. rhodostoma* LAAO (2 mM). The spectral changes occurring during titration of activated *Ca. rhodostoma* LAAO are shown in Fig. 6. These values are higher than those found with the corresponding forms of the *Cr. adamanteus* enzyme (K_d = 63 and 0.068 mM) [10]). Because the formation of a N(5)-sulfite adduct is an indication of the ability of a flavoprotein to stabilize a negative charge at the

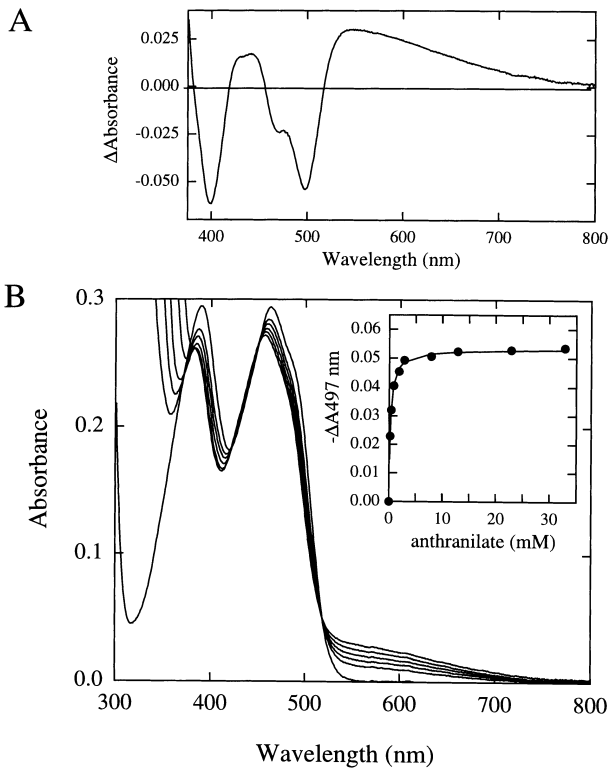


Fig. 7. Titration of activated *Ca. rhodostoma* LAAO with the substrate inhibitor anthranilic acid. (A) Difference spectrum of LAAO in the presence of 32.9 mM anthranilate and uncomplexed protein. (B) Titration experiment; for clarity representative spectra at 0, 0.2, 0.4, 0.9, 2.9 and 22.9 mM anthranilate concentration are depicted (from top to bottom at 497 nm). Insert: plot of the absorbance change at 497 nm as a function of anthranilate concentration. The data points (●) were fitted with a hyperbolic function [f(x) = $A(\Delta 497)_{max}$ × [anthranilate]/K_d × [anthranilate]] yielding a dissociation constant of 0.27 mM.

N(1)–C(2)=O locus of the flavin ring moiety, the much higher dissociation constant of the freeze-inactivated protein can be interpreted in terms of impaired stabilization of the negative charge at this locus. Alternatively, it is also conceivable that the decrease in binding affinity results from reduced accessibility of the sulfite binding site, i.e. the N(5) position of the isoalloxazine ring. In order to distinguish and to further evaluate these possible explanations we have used the competitive substrate inhibitor anthranilate to probe the accessibility of the enzyme active site.

Formation of LAAO–anthranilate complex

Similar to the results described for sulfite binding, it was found that the activated form of LAAO bound anthranilate with a dissociation constant of 0.27 mM (Fig. 7), whereas titration of the freeze-inactivated form with anthranilate did not result in any observable spectral changes up to 8 mM anthranilate. This result is very similar to earlier observations with the *Cr. adamanteus* enzyme [10,23]. As binding of anthranilate to the active site of LAAO is not accompanied by the generation of a negative charge at the N(1)–C(2)=O locus of the flavin ring, it appears a more likely explanation that the reduced binding affinity of the freeze-inactivated form is due to diminished accessibility to the active site of the enzyme.

CONCLUSIONS

In contrast to earlier findings [13], in our hands, *Ca. rhodostoma* LAAO shares the remarkable freeze- and pH-induced reversible inactivation process with LAAOs isolated from *Cr. atrox* and *Cr. adamanteus*. The UV/visible absorbance spectral difference of the freeze- and pH-induced inactive form are very small, suggesting that the two inactive forms provide similar environments for the FAD cofactor. Studies with sulfite and anthranilate indicate that accessibility to the active site is reduced in the inactive forms.

Ophidian LAAOs share 83% sequence identity but except for the highly conserved FAD-binding site sequence, similarity to other LAAOs from bacterial, fungal and algal origin is low. This extended dinucleotide-binding motif is not only present in known FAD-dependent enzymes but also in a number of poorly characterized proteins. Their putative requirement for FAD presents an interesting aspect with regard to their biological activities, e.g. the antibacterial activity of the glycoprotein achacin [24], the antineoplastic activity of the glycoprotein aplysianin A [25] and the unknown role of Cs proteins (the *Cs* gene is part of the Dopa decarboxylase gene cluster) in insects [26].

A most interesting difference among ophidian LAAOs is the number of glycosylation sites: all three ophidian enzymes share a N-glycosylation consensus sequence in the C-terminal part (Asn361 in the *Ca. rhodostoma* sequence). An additional N-glycosylation site was found in *Ca. rhodostoma* LAAO in the N-terminal part of the protein at Asn172. Recently it was reported that glycosylation of *Cr. atrox* LAAO (apoxin I) appears to be involved in the maturation and secretion of active enzyme and possibly also in the apoptosis-inducing effect observed for this enzyme [27]. The crystal structure of *Ca. rhodostoma* LAAO has clearly shown that both N-glycosylation sites are occupied, however, the chemical nature and exact structure of the oligosaccharide(s) have not been investigated in detail. This will be the main focus of future studies.

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