

Catabolic Pathways for Arginine and Methylated Arginines by Plant and Mammalian Copper Amine Oxidases

F. Pintus^a, A. Contini^a, A. Finazzi Agrò^b, G. Floris^a, S. Porcu^a, A. Fais^a, D. Spanò^a and R. Medda^{a,*}

^aDepartment of Applied Sciences in Biosystems, University of Cagliari, Cagliari, Italy

^bDepartment of Experimental Medicine, University of Rome "Tor Vergata", Rome, Italy

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The oxidation of L-arginine, monomethyl L-arginine and asymmetric dimethyl arginine catalyzed by copper/TPQ-amine oxidases from lentil (*Lens esculenta*) seedlings and pig kidney was investigated by optical spectroscopy and HPLC analyses. L-Arginine and its methylated derivatives were shown to be poor substrates for both enzymes and were oxidized by an unusual mechanism yielding glutamate-5-semialdehyde, ammonia, urea and their derivatives as reaction products. These findings suggest that amine oxidases might represent an alternative metabolic pathway of the arginine and its methylated derivatives, yielding new metabolites like urea, methylurea and dimethylurea.

Keywords: Amine oxidase, Arginine, Asymmetric dimethyl arginine, *Lens esculenta*, Pig kidney

INTRODUCTION

L-Arginine is an amino acid that has numerous functions in animal and plant kingdom. In mammals, arginine is used to make very active compounds such creatine, L-glutamate, and L-proline, and can be converted to glucose and glycogen if needed. In plants arginine residues form a significant store of nitrogen in seeds. The degradation of arginine releases this nitrogen supporting the growth of the seedlings during germination.

Among various enzymes involved in metabolic pathways for arginine catabolism are:

- i.* Arginase (E.C. 3.5.3.1) that plays a degradative role of arginine yielding urea and ornithine.
- ii.* Arginine decarboxylase (E.C. 4.1.1.19) that produces agmatine from arginine.
- iii.* Nitric oxide synthase (E.C. 1.14.13.39) that, acting on

arginine, yields N^G-hydroxyarginine as the first product, and then citrulline and nitric oxide.

While in animals arginine can be considered as the natural substrate of nitric oxide synthase, and the metabolism and role of NO has been thoroughly investigated, the source of NO in plants has been the subject of much debate. Both arginine and nitrite-dependent pathways have been shown to be precursors of NO. In plants NO plays an important role in growth and development and is mainly involved in many different processes such as seed germination, root development,

Abbreviations: ADMA, asymmetric dimethyl arginine; CAOs, copper/TPQ amine oxidases; DDAH, dimethylarginine-dimethylaminohydrolase; LSAO, lentil seedling copper/TPQ-amine oxidase; MMA, monomethyl L-arginine; PKAO, pig kidney copper/TPQ-amine oxidase; SDMA, symmetric dimethyl arginine; TPQ, 6-hydroxydopa quinone; TPQ_{aq}, Cu^{II}-aminoquinol; TPQ_{sq}, Cu^I-semiquinolamine radical.

*Corresponding author. E-mail: rmedda@unica.it

stomatal closure and the expression of defence-related gene against pathogens [1].

Monomethylarginine (MMA) and symmetric and asymmetric dimethylarginines (SDMA and ADMA) have recently gained ground as they result from post-synthetic modification of arginyl residues in proteins, particularly those involved in the regulation of gene expression [2]. These active compounds, formed by the action of arginine methyl transferases I and II (E.C. 2.1.1.125, 2.1.1.126) using S-adenosyl methionine as methyl donor [2], are released after proteolysis and may enter the circulation [3].

In mammals MMA, ADMA, and SDMA are known to be inhibitors of NO synthase [4]. In particular MMA and ADMA act as competitive inhibitors at the active site of the enzyme, whereas SDMA may indirectly inhibit NO synthesis by interfering with arginine uptake [5]. Methyl arginines are either eliminated as such through the kidney or metabolized by hydrolytic enzymes. For instance, ADMA is hydrolyzed by dimethylarginine-dimethylaminohydrolase (E.C. 3.5.3.18; DDAH) to citrulline and dimethylamine [6].

The presence and role of MMA, ADMA and SDMA in plants has not yet been reported. Amine oxidases (amine oxygen oxidoreductase, deaminating, copper/TPQ containing; EC 1.4.3.6; CAOs), a widespread class of enzymes, are present in all living systems. These enzymes are characterized by the presence of two redox centers, a divalent copper and the quinone of 2,4,5-trihydroxyphenylalanine (TPQ) derived by a post-synthetic modification of a tyrosinyl residue of the protein [7,8]. Plant CAOs from various species have been purified to homogeneity and characterized, the best known and studied being those from lentil (*Lens esculenta*) [9] and pea (*Pisum sativum*) [10] and from latex of the shrub *Euphorbia characias* [11]. In mammals, the best known enzymes are those from pig kidney and from bovine serum [12-14].

The physiologic role of CAOs has been debated for several years and there is still controversy about their significance despite their diffusion in all living organisms and in many different tissues. These enzymes control the level of very active compounds, *i.e.* mono-, di- and polyamines. The oxidation of these compounds may generate other biologically active substances like aldehydes, ammonia and hydrogen peroxide. Although the physiological role of CAOs is not exactly known, in mammals these enzymes might be involved

in detoxification and in metabolic and vascular diseases, and in plants are implicated in wound healing, detoxification and cell growth. Moreover, the aldehyde products might have a key role in the biosynthesis of some alkaloids [15], and the hydrogen peroxide, that is always formed in the reactions catalyzed by CAOs, is considered either a crucial substrate for important biochemical processes, or a signal and a defense molecule rather than a noxious waste product.

In our previous paper [16], we reported that the amine oxidase from lentil seedlings (LSAO) was able to oxidize arginine by an unusual mechanism yielding glutamate-5-semialdehyde, ammonia and urea as reaction products. In this paper, we have investigated whether CAOs might be able to oxidize, besides arginine, its methylated derivatives. We found that, indeed two different amine oxidases from animal (pig kidney amine oxidase, PKAO) and plant (LSAO) kingdoms can oxidize MMA and ADMA besides arginine, yielding as products of the reactions, glutamate-5-semialdehyde, ammonia, hydrogen peroxide, and respectively, methylurea and dimethylurea. An alternative pathway of the catabolism of arginine and its methyl-derivatives in both animal and plant kingdom is proposed.

EXPERIMENTAL

Reagents

L-Arginine, MMA, ADMA, SDMA, urea, methyl urea, dimethyl urea, 1-amino-4-guanidinobutane (agmatine), N^G-hydroxyarginine, L-citrulline, 4-hydroxy-3-methoxy phenylacetic acid, α -oxoglutarate acid disodium salt, β -NADH disodium salt (preweighed vials), urease, horseradish peroxidase type VI, and glutamate dehydrogenase type III from bovine liver were purchased from Sigma (St Louis, MO, USA) and used without further purification. LSAO was purified according the procedure reported [17]. The concentration of the purified enzyme (two copper ions and a $M_r = 150,000$) was determined using an $\epsilon_{498} = 4100 \text{ M}^{-1} \text{ cm}^{-1}$ or an $\epsilon_{278} = 2.45 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. PKAO was purified according to the procedure previously described [12]. The concentration of the purified enzyme (two copper ions and a $M_r = 180,000$) was determined using an ϵ_{490} of $4000 \text{ M}^{-1} \text{ cm}^{-1}$ or an ϵ_{278} of $3 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Amine Oxidase Activity

The K_m value for arginine, MMA and ADMA was obtained from a double reciprocal plot in 100 mM K-phosphate buffer (pH 7.0) at 37 °C. Catalytic activity (k_{cat}) and the k_{cat}/K_M values were compared to those of the best substrate, putrescine (LSAO) or cadaverine (PKAO).

Oxygen uptake was determined polarographically at 37 °C by an oxygraph (Hansatech Instruments LTD, Norfolk, UK) equipped with a Clark electrode. The standard reaction mixture (1 ml) contained the enzyme in 100 mM K-phosphate buffer (pH 7.0). The reaction was started by addition of substrate after at least 10 min pre-incubation.

Determination of Reaction Products

Hydrogen peroxide was determined by the peroxidase:4-hydroxy-3-methoxy phenylacetic acid method [18]. Ammonia production was determined by the amount of NADH consumed in the presence of glutamate dehydrogenase and α -oxoglutarate acid using an $\epsilon_{340} = 6200 \text{ M}^{-1} \text{ cm}^{-1}$, and urea was determined as ammonia production after the reaction with urease when arginine was used as substrate.

Reaction of LSAO and PKAO with Arginine Derivatives

Either arginine or MMA or ADMA (1 mM) were added to 1 ml of 100 mM K-phosphate buffer (pH 7.0) containing (10 μM) LSAO. After 6 h of incubation at 37 °C, the three enzymatic reactions were stopped by heat inactivation (97 °C for 5 min). The incubates were centrifuged at 18,000 rpm for 15 min and the precipitate discarded. Aliquots of supernatants were diluted with ultrapure water and analyzed by the following methods:

HPLC analysis. Arginine, MMA, ADMA, urea, monomethyl urea and dimethyl urea were separated by HPLC chromatography. In this system, arginine ($R_t = 2.75$ min), MMA ($R_t = 2.94$ min), ADMA ($R_t = 3.30$ min), urea ($R_t = 3.22$ min), monomethyl urea ($R_t = 3.10$ min) and dimethyl urea ($R_t = 4.05$ min) were easily distinguishable. The separation was made using an HPLC chromatography Agilent 1100 series equipped with Zorbax 300 SB-C18 5- μm particle size, 250 \times 4.6 mm ID column. The mobile phase was an aqueous KH_2PO_4 25 mM buffer. The method was based on isocratic elution. The column effluent was monitored through

diode array UV detector at 210 nm.

Capillary electrophoresis coupled with Mass spectrometry (CE-MS). Monomethyl urea, dimethyl urea and Δ^1 -pyrroline-5-carboxylic acid, derived from cyclization of glutamate-5-semialdehyde, were detected by CE-MS. Capillary Electrophoresis automated apparatus was obtained from Agilent Technologies (Waldbronn, Germany) equipped with diode array UV detector and external nitrogen pressure. The CE apparatus was coupled with the Esquire 3000 plus mass spectrometer (Bruker Daltonics, Bremen, Germany) via a coaxial sheath liquid electrospray ionization (ESI) interface (Agilent Technologies, Waldbronn, Germany). The sheath liquid was delivered by an external syringe pump (Cole Palmer, Vernon Hills, Illinois, USA) at a constant flow rate of 240 $\mu\text{l h}^{-1}$. Nebulizing and drying gas (nitrogen) were set at 5.0 psi and 5.0 l min^{-1} , respectively. Dry gas temperature was 300 °C. Mass spectrometry capillary voltage was 4500 V. Separations were performed in 50 μm I.D., 375 μm O.D. fused silica uncoated capillaries (Composite Metal Services, Hallow, Worcs., UK) of total length of 89 cm. Effective length was 21.5 cm for UV detection and 89 cm for MS detection. The CE-MS apparatus was used both for analytes identification using capillary electrophoresis separation method and direct sample injection mode (flow injection analysis, FIA).

Mass spectrometry detection was performed in positive polarity mode both in full scan and tandem mass spectrometry (MS^2) product ion scan modes. In MS^2 detection an isolation width of ± 4.0 m/z and fragmentation amplitude of 1.0 V in positive ionization and normal resolution scan was used. The acquisition of the full scan or MS^2 extracted ion current (EIC) signals was made in 50-230 m/z mass range using a maximum accumulation time of 100 ms and a set target value of 50,000 and by activating the ion charge control (ICC) function.

The temperature of the CE-MS assembly cartridge was set at 25 °C. The CE running voltage was 23 kV (positive polarity). Samples were injected at the anodic end at 50 mbar \times 10 followed by BGE injection at 50 mbar \times 15 s. Formic acid solutions at 1.5 M was used as BGE and was daily prepared. Water/methanol (30:70, v/v) mixture containing 0.1% formic acid was used as sheath liquid solution.

All kinetic parameters and the amount of reaction products formed were calculated as the mean of at least five different measurements.

Spectrophotometric Methods

Absorption spectra were recorded at room temperature in a Ultrospec 2100 spectrophotometer (Biochrom Ltd., Cambridge, UK). Anaerobic experiments were made after several cycles of evacuation followed by flushing with O₂-free argon at 25 °C in a Thunberg-type spectrophotometric cuvette (Soffieria Vetro, Sassari, Italy) in which anaerobic additions of various reagents could be made through a rubber cap with a syringe.

RESULTS AND DISCUSSION

Activity of LSAO and PKAO on Arginine and its Derivatives

As previously reported [16], LSAO was able to oxidize L-arginine at low rate. In 100 mM K-phosphate buffer (pH 7.0) at 37 °C the K_m value was found to be 32 mM and the k_c value was about five orders of magnitude lower than that observed with putrescine ($2.8 \times 10^{-3} \text{ s}^{-1}$ vs. 155 s^{-1} ; (Table 1). Through the experiments performed in this work we showed that LSAO was also able to oxidize arginine derivatives with k_c values, for MMA and ADMA, of $1.9 \times 10^{-3} \text{ s}^{-1}$ and $8 \times 10^{-4} \text{ s}^{-1}$ respectively, and a K_m value of 41 mM for both the substrates (Table 1).

Also PKAO was able to oxidize L-arginine. In 100 mM K-phosphate buffer (pH 7.0), at 37 °C, the K_m value for arginine as substrate was calculated to be 13 mM. The k_c value was about three orders of magnitude lower than observed with cadaverine ($3.4 \times 10^{-3} \text{ s}^{-1}$ vs. 4.5 s^{-1} ; (Table 1). PKAO was also able to oxidize arginine derivatives with k_c values, for MMA and ADMA, lower than that obtained with arginine ($5 \times 10^{-5} \text{ s}^{-1}$ for MMA and $9 \times 10^{-5} \text{ s}^{-1}$ for ADMA; Table 1), and even lower than that obtained with LSAO. Due to the very low turnover rate of the reactions of PKAO with methylated arginines, we were unable to determine the K_m value for arginine derivatives (Table 1) despite using large amounts of enzyme ($\geq 10 \mu\text{M}$).

SDMA, which has one methyl group on each guanidine nitrogen, was not substrate for LSAO and PKAO. This is not surprising since these enzymes require at least one free amino group for substrate recognition.

Regarding the reactions between LSAO or PKAO with arginine, MMA and ADMA, the stoichiometry was similar to that observed with other substrates (putrescine or cadaverine), yielding 1 mol of ammonia and hydrogen peroxide per mole of oxygen consumed. Arginine oxidation by either CAO yielded a fourth product, namely urea. The production of urea was determined first by HPLC analyses and was confirmed by

Table 1. Kinetic Parameters of LSAO (a) and PKAO (b) Using Putrescine, Cadaverine, L-Arginine, MMA and ADMA as Substrates. Buffer Used: 100 mM K-Phosphate Buffer (pH 7.0). Data Represent the Mean of Five Different Measurements

	$k_c \text{ (s}^{-1}\text{)}$	$K_m \text{ (mM)}$	$k_c/K_m \text{ (mM}^{-1} \text{ s}^{-1}\text{)}$
Putrescine	$155 \pm 12 \text{ (a)}$	0.24 (a)	645 (a)
Cadaverine	$4.5 \pm 0.13 \text{ (b)}$	0.1 (b)	45 (b)
L-Arginine	$2.8 (\pm 0.1) \times 10^{-3} \text{ (a)}$	32 (a)	$8.7 \times 10^{-5} \text{ (a)}$
	$3.4 (\pm 0.1) \times 10^{-3} \text{ (b)}$	13 (b)	$2.6 \times 10^{-4} \text{ (b)}$
MMA	$1.9 (\pm 0.4) \times 10^{-3} \text{ (a)}$	41 (a)	$4.4 \times 10^{-5} \text{ (a)}$
	$5 (\pm 0.9) \times 10^{-5} \text{ (b)}$	nd	-
ADMA	$8 (\pm 0.7) \times 10^{-4} \text{ (a)}$	41 (a)	$1.9 \times 10^{-5} \text{ (a)}$
	$9 (\pm 1.2) \times 10^{-5} \text{ (b)}$	nd	-

k_c = catalytic-center activity defined as (moles of substrate consumed)/(moles of active sites) s^{-1} . nd = not detectable.

addition of urease at the end of the reaction, which liberated 2 further moles of ammonia per mole of L-arginine.

Since methylated urea derivatives were not substrates for urease, the production of methylurea and dimethylurea was determined by HPLC and CE-MS analyses. The samples were performed as described in Experimental section (Reaction of LSAO and PKAO with arginine derivatives). Aliquots of supernatants were diluted with ultrapure water and the products were subsequently analyzed by 20 μ l injection of reaction mixture onto a reverse phase HPLC column. A peak with retention time of 3.10 and 4.05 min was obtained after incubation with MMA and ADMA, respectively, indicative of the formation of monomethyl urea and dimethyl urea (Fig. 1A and 1B).

CE-MS experiments were carried out in order to further characterize the products of the reactions of LSAO or PKAO either on arginine, or on MMA or on ADMA. CE-MS analyses afforded detecting, in the three incubation solutions, a common product of 114.3 D corresponding to the $[M+H]^{1+}$ mass value of the cyclization product of glutamate-5-semialdehyde (Scheme 1e). Moreover, in the incubation solution of CAOs with MMA, the $[M+H]^{1+}$ mass value of 75.7 D of methylurea, and in the incubation solution of CAOs with ADMA, the $[M+H]^{1+}$ mass value of 89.5 D of dimethylurea were detectable. In the incubation solution of CAOs with arginine the $[M+H]^{1+}$ mass value of urea (theor. 61.0 D) was not detected, probably for the poor ionization ability of the urea molecule under the ESI conditions applied (results not shown).

It is well known that CAOs are fully inactivated by the carbonyl reagent phenylhydrazine [19]. Phenylhydrazine-treated LSAO or PKAO were indeed completely inactive toward arginine and its derivatives, and neither urea nor ornithine was detected at the end of the experiment excluding a possible contamination of the samples by arginase. A contamination by arginine decarboxylase, which produces agmatine from arginine, or by nitric oxide synthase which, acting on arginine, yields N^G -hydroxyarginine as the first product and then citrulline and nitric oxide [20], was similarly excluded because neither agmatine nor N^G -hydroxyarginine and citrulline were detected at the end of the experiment. Moreover, it is worth recalling that MMA and ADMA, potent inhibitors of NO synthase [4], were shown to be substrates for

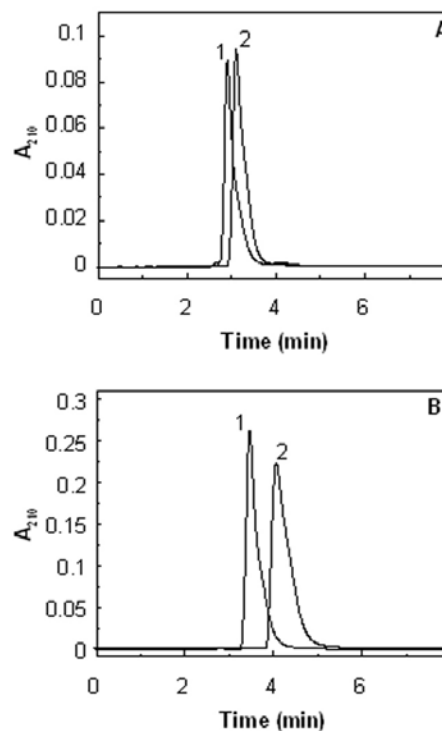


Fig. 1. Reverse phase HPLC separation of monomethyl L-arginine and asymmetric dimethyl arginine oxidation products generated by pig kidney amine oxidase. PKAO (10 μ M) was incubated with 1 mM MMA (A) or ADMA (B) for 6 h, in 1 ml of 100 mM K-phosphate buffer (pH 7.0) at 37 $^{\circ}$ C and the reaction products were separated and analyzed as described under “Experimental”. A: (1) MMA reference standard (Rt = 2.94 min) and (2) monomethyl urea (Rt = 3.10 min) obtained after 6 h incubation of PKAO with MMA. B: (1) ADMA reference standard (Rt = 3.30 min) and dimethyl urea (Rt = 4.05 min) obtained after 6 h incubation of PKAO with ADMA.

both enzymes, LSAO and PKAO.

Finally, the absence of citrulline after the reaction by CAOs and ADMA confirmed that no contamination by DDAH in the enzyme preparation occurred.

The presence of citrulline, N^G -hydroxyarginine and agmatine were excluded from the incubation mixture by both thin-layer chromatography [16] and by HPLC analyses (not shown).

Spectrophotometric Experiments

The visible spectrum of resting LSAO shows a typical broad absorption centered at 498 nm [21]. When L-arginine, MMA or ADMA (10 mM) were added to LSAO in anaerobiosis, there was a lag time before the disappearance of the 498 nm band, indicating that the formation of the bleached Cu^{II} -aminoquinol (TPQ_{aq}) was slow. Afterwards, new absorbance bands centered at 360, 434 and 464 nm appeared conferring a yellow color to the solution. This absorption spectrum was due to the generation of a Cu^{I} -semiquinolamine radical species (TPQ_{sq}) [21] confirming that, as previously observed on the reaction between LSAO and arginine [16], the oxidation of MMA and ADMA followed the same mechanistic pathway already reported for other substrates. The semiquinone radical, which has a distinguishable absorption and EPR spectra, forms because of the transfer of one electron from the reduced TPQ to the copper atom, thus reduced to Cu^{I} . The radical species reached its maximum concentration after about 4 h (Fig. 2A).

Mammalian CAOs do not normally populate the Cu^{I} -semiquinolamine radical [22]. When arginine, MMA or ADMA were added to PKAO in anaerobiosis, the broad absorption band at 490 nm started disappearing after about 15 min, indicating again the slow conversion of the TPQ cofactor to a bleached TPQ_{aq} species (Fig. 2B).

In both enzymes the readmission of oxygen restored the absorption spectrum of oxidized TPQ and the enzymes turned fully active.

A possible mechanism for the oxidation of arginine and its methylated derivatives by CAOs is reported in Scheme 1. Here, the formation of the adduct between TPQ and arginine(s) is shown involving the protonated imino nitrogen of the guanidinium group, and experiments performed with monomethyl L-arginine and the asymmetric dimethyl arginine which demonstrate that the mechanism is the only feasible one. The transfer of two electrons from the substrate to the cofactor forms the hydroquinol derivative.

Similar to that observed using fast substrates (putrescine or cadaverine), the reoxidation of the reduced aminoquinol (after the reaction with arginine and its methylated derivatives) in plant and mammalian CAOs occur via two different pathways: in plants a specific semiquinone radical intermediate is at work whereas, in mammalian the aminoquinol undergoes direct reoxidation by molecular oxygen.

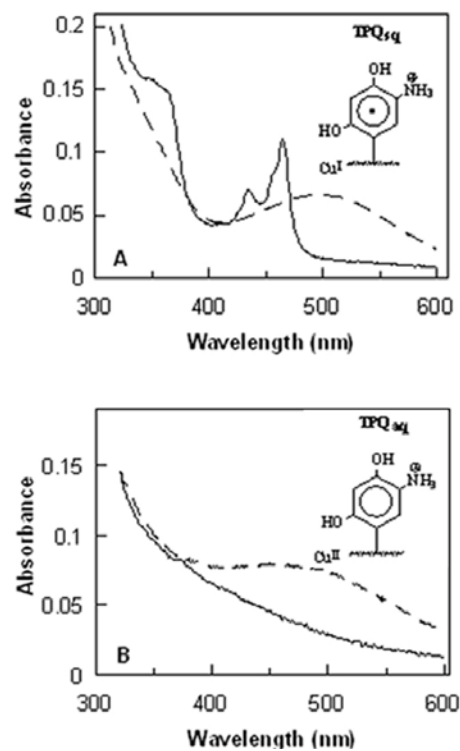
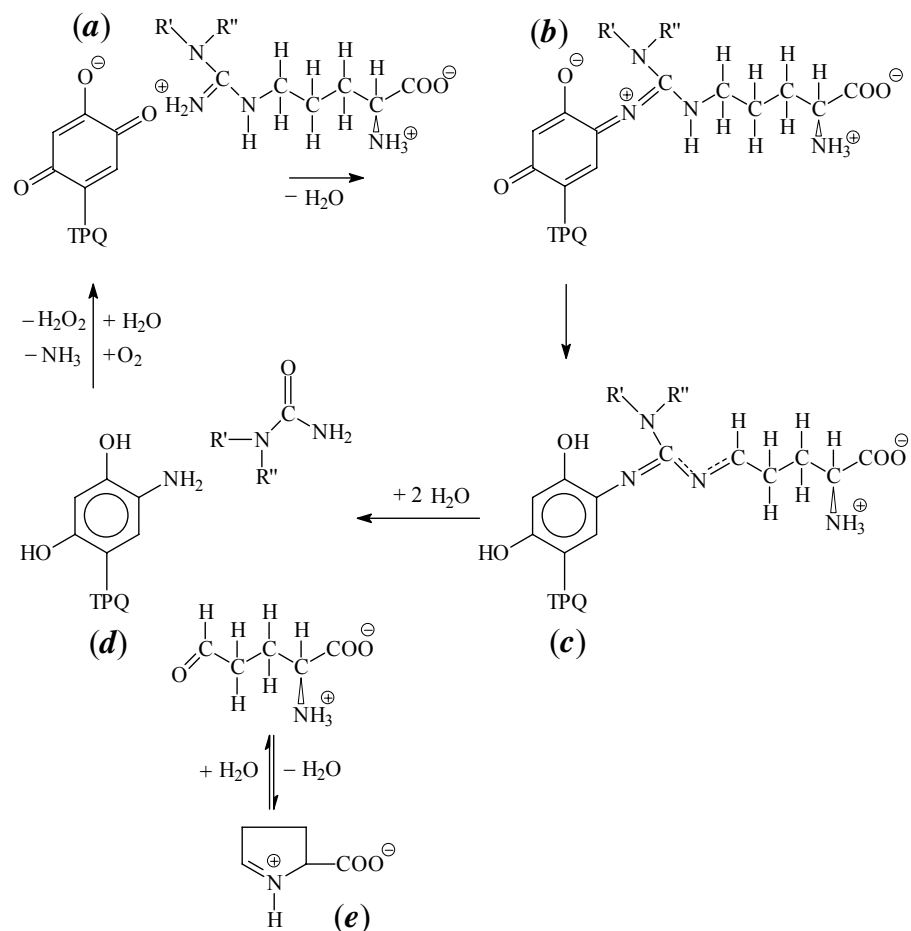


Fig. 2. (A) Native LSAO (16 μM) in 100 mM K-phosphate buffer (pH 7.0), under anaerobic conditions before (---) and 240 min after (—) addition of 10 mM MMA, showing the formation of the Cu^{I} -semiquinolamine radical (TPQ_{sq}). (B) PKAO, 19 μM , in 100 mM K-phosphate buffer (pH 7.0), before (---) and 240 min after (—) addition of MMA in anaerobic conditions, showing the formation of the Cu^{II} -aminoquinol.

In conclusion, LSAO and PKAO are able to oxidize L-arginine and its derivatives MMA and ADMA. Both enzymes oxidized arginine by a new mechanism involving the consumption of 1 mol of oxygen and the production of 1 mol of ammonia, 1 mol of hydrogen peroxide, 1 mol of urea, and 1 mol of glutamate-5-semialdehyde per mole of substrate. MMA and ADMA are oxidized in a similar manner, but, instead of urea, methylurea and dimethylurea are formed respectively.

In view of the very low activity shown by these enzymes toward arginine, MMA and ADMA, it is difficult to postulate a definite physiological role for this newly discovered activity of CAOs. Furthermore, these substrates can be attacked much more efficiently by other enzymes like arginase, arginine

Copper-Amine Oxidases and Arginine-Derivatives



Scheme 1. Oxidation of arginine (R' and $R'' = H$), monomethyl L-arginine ($R' = H$; $R'' = CH_3$) and asymmetric dimethyl arginine (R' and $R'' = CH_3$) by amine oxidases. (a) Cu^{II} -resting oxidized enzyme; (b) Cu^{II} -substrate Schiff base; (c) Cu^{II} -product Schiff base; (d) Cu^{II} -aminoquinol.

decarboxylase, NO synthase and DDAH. Nevertheless, the arginine degradation pathways can be accounted for by CAOs. It is worth recalling that arginase plays a degradative role on arginine yielding urea and ornithine. The latter is a good substrate for both LSAO and PKAO yielding glutamate-5-semialdehyde. On the other hand, arginine decarboxylase produces agmatine from arginine, and again agmatine is a good substrate for PKAO and LSAO [23]. Moreover, arginine and ornithine are precursors for the synthesis of putrescine, spermine and spermidine, and these di- and polyamines are indeed very good substrates for CAOs.

Finally, ADMA and, at a lesser extent MMA, are inhibitors of NO synthase. Thus, a possible significance of CAOs might

be the removal of ADMA and MMA; however, it appears unlikely since DDAH is much more active toward these substrates. The basic difference between the action of the ADMA-lytic enzymes is the nature of the products. DDAH generates citrulline and dimethylamine, whereas CAOs give rise to the four different products glutamate-5-semialdehyde, ammonia, hydrogen peroxide and asymmetric dimethylurea.

The first three products may derive much more efficiently than by other metabolic routes; while asymmetric dimethylurea, a powerful antioxidant particularly reactive with hydroxyl radicals [24], is not formed by any physiological pathway known to date.

Thus, the ability of different CAOs from plants and

animals to oxidize these molecules, and the presence of CAOs in many different tissues, suggest that this activity may not be due to chance. Accordingly, the physiological role of this reaction warrants further “*in vivo*” and “*ex vivo*” investigation.

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