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LENTIL SEEDLINGS AMINE OXIDASE: PREPARATION AND PROPERTIES OF THE COPPER-FREE ENZYME

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SUMMARY: The reaction of copper-free lentil seedlings amine oxidase with substrates has been studied. While devoid of catalytic activity, this enzyme preparation is still able to oxidize two moles of substrate and to release two moles of aldehyde and two moles of ammonia per mole of dimeric protein. The same stoichiometry has been determined on the native enzyme in the absence of oxygen. Although copper is essential for the reoxidation of the reduced enzyme, a binding of oxygen to the copper-free protein has been demonstrated.

Diamine oxidases (E.C.1.4.3.6, deaminating) are enzymes found in all forms of life (1). They catalyze the oxidation of one primary amino group of the substrate according to the following reaction:

$$R\text{-}CH_2NH_2 + O_2 + H_2O = R\text{-}CHO + NH_3 + H_2O_2$$

It is generally agreed that the reaction is a two-step process involving first the reduction of the enzyme by the substrate and then the oxidation of the reduced enzyme by oxygen (2-4). All diamine oxidases from eukaryotic organisms contain cupric copper, essential for the catalytic activity (1 -5), and a second cofactor whose nature has not yet been established (6). The role of copper is still to be defined since it neither binds the substrate (7) nor undergoes redox reactions (7-11). Recently a diamine oxidase from lentil seedlings (LSAO) has been purified to homogeneity (12). This

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enzyme is very similar to other plant amine oxidases (13-15) and particularly to pea seedlings amine oxidase (16). The latter enzyme was also prepared in the copper-free form (16). In the present paper we report the preparation of copper-free LSAO and the stoichiometry of its reaction with substrates.

EXPERIMENTAL PROCEDURES

All the chemicals used were of the highest commercial grade. LSAO was purified from the seedlings of lentil (Lens esculenta) as reported by Floris et al. (12). Protein concentration was determined by the absorbance at 278 nm (ε = 2.00 x 10^5 M^{-1} cm^{-1}). Enzymic activity was determined polarographically as described previously (12).

Preparation of copper-free LSAO. Copper-free LSAO was prepared as follows. An enzyme solution was dialyzed extensively against bidistilled water at 4°C. The dialyzed solution was made 0.01 M in sodium diethyldithiocarbamate and allowed to stand at 4°C for 36 h, then centrifuged at 105,000 x g for 2 h. The orange supernatant was dialyzed extensively against bidistilled water and then centrifuged at 15,000 rpm for 30 min. The precipitate was discarded and the pink supernatant stored at -20°C if not immediately used.

Anaerobic experiments. Anaerobic experiments were conducted at 25°C in a Thunberg-type spectrophotometric cuvette where anaerobic additions of various reagents can be made with a syringe through a rubber cap.

Ammonia determination. Ammonia production was determined spectrophotometrically in the coupled glutamate dehydrogenase assay following the oxidation of NADH (17).

Aldehyde determination. Aldehyde release from putrescine was determined spectrophotometrically by reaction with o-aminobenzaldehyde (o-ABA) (18). Authentic Δ^1-pyrroline was added to test the system. Alternatively the aldehyde formed was determined by the method of Naik et al. (19) after denaturation of the enzyme solution with TCA (final concentration 10%). When p-DABA (p-dimethylaminomethyl-benzylamine) was used as the substrate, aldehyde production was determined by O.D. change at 250 nm using ε =11,000 M^{-1} cm^{-1} (20).

RESULTS

Removal of copper from LSAO. Copper is almost completely removed from LSAO by treatment with diethyldithiocarbamate, as described in Materials and Methods. The residual copper, determined by atomic absorption spectroscopy, is 0.8 % of the original content. The copper-free protein samples are

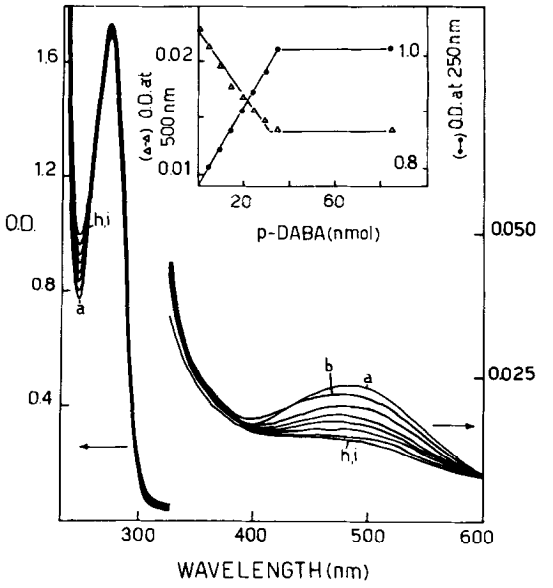

Fig. 1
Titration of copper-free LSAO with p-DABA.
2 ml of 8.7 x 10^{-6} M copper-free LSAO in 0.1 M phosphate buffer, pH 7, were added with 5 µL aliquots of 1 x 10^{-3} M p-DABA in aerobiosis. Spectra were recorded before (a) and immediately after (b-h) each addition and did not show variations at longer times. Spectrum i was recorded after addition of 5 µL of 1 x 10^{-2} M p-DABA.
Inset: stoichiometry of the reaction followed at 250 (●) and 500 (△) nm. The substrate/enzyme molar ratio at the end point is 2.1 and 1.8, respectively.

stable, practically devoid of enzymic activity and do not show any EPR signal. The addition of less than stoichiometric amounts of cupric copper under controlled conditions restores the enzymic activity in proportion to the copper added. After dialysis against EDTA the reconstituted LSAO shows an EPR spectrum identical to that of the native enzyme.

Copper-free LSAO is pink, showing a broad absorption peak in the visible at 480 nm (fig. 1), i.e. shifted toward shorter wavelengths with respect to the native enzyme, but with similar intensity (ε = 2600 M^{-1} cm^{-1}). No difference in the uv region between the copper containing and the copper-free enzyme is apparent, both showing a maximum at 278 nm.

TABLE I
Stoichiometry of products formation by LSAO

LSAO	O_2	Substrate	Products (mol/mol dimer)	
			Aldehyde	Ammonia[a]
native	-	putrescine	0[b]	0
	-	putrescine	1.88 (3)[c]	---
	-	p-DABA	1.96 (5)[d]	0
copper-free	+	putrescine	1.88 (1)[b]	1.96 (2)
	+	p-DABA	1.84 (2)[d]	1.78 (1)
	-	putrescine	0[b]	0
	-	p-DABA	1.84 (2)[d]	0

a) determined by reaction with glutamate dehydrogenase;

b) determined by reaction with o-ABA;

c) determined according to Naik et al. (19) after TCA denaturation;

d) determined from absorbance increase at 250 nm.

Values are the average of the number of experiments indicated in brackets. For experimental details see text.

Reaction of LSAO with substrates and analogues. The addition of excess putrescine to native LSAO in air is accompanied by a rapid bleaching of the visible absorption. This absorption slowly reappears after exhaustion of the substrate. A similar behaviour is obtained with the copper-free enzyme, but in this case the absorption band is not restored even after dialysis. Furthermore the full bleaching of the absorption band only requires two moles of substrate per mole of copper-free enzyme (Fig. 1 and Table I).

If substrate is added in anaerobiosis to the native LSAO a different pattern is observed (fig. 2). In fact the addition of putrescine is followed by the disappearance of the 490 nm absorption band and by the appearance of an absorption band at 350 nm and two sharp bands at 460 and 430 nm. Other substrates like cadaverine, spermidine and the analogue p-DABA produce the same bands when added to LSAO in the ab-

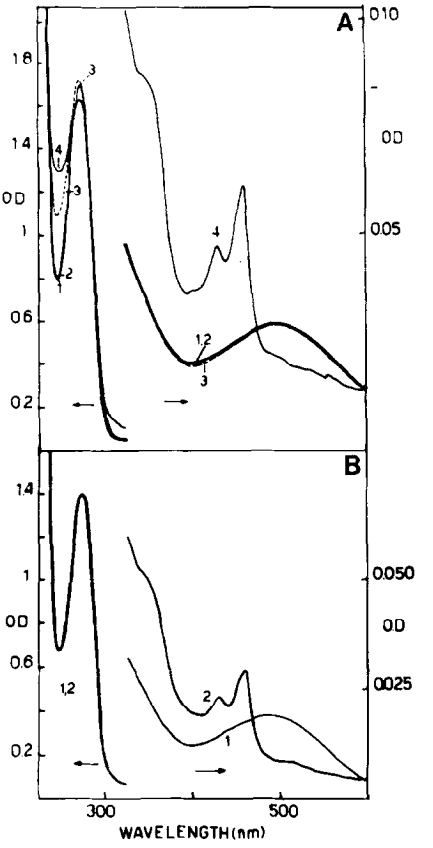

Fig. 2
Reaction of LSAO with substrates.
A: Absorption spectra of 8.5 x 10^{-6}M LSAO in 0.1 M phosphate buffer, pH 7, 1) before and 2) after deoxygenation, 3) after anaerobic addition of p-DABAl (final concentration 2.2x10^{-5}M), 4) after anaerobic addition of p-DABA (final concentration 2.5x10^{-4}M) to 3).
B: Absorption spectra of 7.2 x 10^{-6}M LSAO in 0.1 M phosphate buffer, pH 7, in anaerobic conditions.
1) no addition; 2) after addition of putrescine (final concentration 1.7 x 10^{-2}M).

sence of air. Two moles of p-DABA per mole of native enzyme are needed to obtain maximum 460 nm absorption.

Formation of products in anaerobiosis. Amine oxidases have long been known to follow a ping-pong reaction mechanism. In the first half-reaction aldehyde and ammonia are released (2,4,21,22).

At difference with other amine oxidases, no aldehyde release from native or copper-free LSAO is observed in anae-

robiosis using putrescine as a substrate, unless the reacted enzymes are anaerobically denatured with TCA. When p-DABA is used as the substrate an increase in absorbance at 250 nm, due to the aldehyde formed (20), is observed even in the absence of air. The formation of two moles of aldehyde per mole of enzyme is determined with each substrate (Fig. 1 and Table I).

Ammonia is released only after admission of air. Again the formation of two moles of NH_3 per mole of enzyme is observed with LSAO and copper-free LSAO. Due to the analytical method used it was impossible to determine the release of NH_3 in anaerobiosis under denaturing conditions.

DISCUSSION

Copper can be reversibly removed from LSAO without affecting significantly the visible absorption of the enzyme. The addition of substrate to copper-containing or copper-free LSAO in air completely bleaches this absorption. Native LSAO treated with substrates in anaerobiosis becomes bright yellow due to the formation of two sharp absorption bands at 430 nm and 460 nm. These peaks gradually fade out upon admission of air and eventually disappear. They are related to the enzyme since they have identical shape and position with every substrate used. The titration with substrate suggests the presence of two active sites per LSAO dimer, i.e. one per copper atom. The presence of two active sites is also supported by the stoichiometry of product formation (Table I). This finding is at variance with the stoichiometry reported for all known Cu-amine oxidases, with the exception of bovine plasma and Aspergillus Niger amine oxidases (17, 22, 24). The fungal enzyme, however, also binds two moles of phenylhydrazine per mole of enzyme, while all the others,

included LSAO (23), bind only one. Copper is not needed for the formation of aldehyde or ammonia since they are formed also by copper-free LSAO. The metal is instead essential for the reoxidation step. In fact the copper-free enzyme does not recover its color after reacting with the substrate. Furthermore the copper-free enzyme does not form the yellow intermediate when treated with substrate in the absence of air.

Surprisingly copper-free LSAO requires the presence of oxygen to release ammonia. This observation may be connected with the finding that the substrate-bleached form of native LSAO does not react with phenylhydrazine in the absence of air. Upon oxygenation the enzyme is slowly but completely converted to the characteristic adduct absorbing at 435 nm (23). Copper-free LSAO reacts in quite the same way. Thus a binding of oxygen to the organic moiety responsible for the visible absorption may be proposed. Work is in progress to identify this organic cofactor and to check by rapid mixing experiments the time course of enzyme bleaching and aldehyde production.

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