Oxidation of Spermine by an Amine Oxidase from Lentil Seedlings

Antonina Cogoni, Alessandra Padiglia, Rosaria Medda, Paolo Segni, and Giovanni Floris*

Istituto di Chimica Biologica, Università di Cagliari, via della Pineta, 77 09125 Cagliari, Italy

ABSTRACT

Spermine is a substrate of lentil (Lens culinaris) seedling amine oxidase and the oxidation products are reversible inactivators of the enzyme. The spermine is oxidized at the terminal amino groups to a dialdehyde: 2 moles of hydrogen peroxide and 2 moles of ammonia per mole of spermine are formed. The pH optimum of the enzyme with spermine is 7.9 in Ti-HCl buffer; the $K_m$ value is $4.4 \times 10^{-4}$ molar, similar to that found with other substrates (putrescine and spermidine).

The polyamines spermidine and spermine and the diamine putrescine which appear to be ubiquitous in animals and plants may be involved in the regulation of growth. Tabor et al. (14) proposed that the plasma amine oxidase oxidizes spermine and spermidine only at terminal amino groups with the formation of the corresponding aldehydes, according to the following equations:

\[
\text{NH}_2-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{NH} + 2 \text{ O}_2 + 2 \text{ H}_2\text{O} \\
\rightarrow \text{OHC-CH}_2-\text{CH}_2-\text{NH}-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{CH}_2-\text{CHO} + 2 \text{ NH}_3 + 2 \text{ H}_2\text{O}
\]

\[
\text{NH}_2-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{NH} + \text{ O}_2 + \text{ H}_2\text{O} \\
\rightarrow \text{OHC-CH}_2-\text{CH}_2-\text{NH}-(\text{CH}_2)_2-\text{NH} + \text{ NH}_3 + \text{ H}_2\text{O}. 
\]

Alarcon (1) concluded that acrolein is formed during the enzymatic oxidation of spermine and spermidine by plasma amine oxidase, probably by the spontaneous decomposition of the intermediate products, the unstable aminoaaldydehydes. Kimes and Morris (8) described a procedure for the isolation of mono-oxidized and di-oxidized spermine in relatively pure form during spermine oxidation by plasma amine oxidase: β-elimination yielded acrolein and aldol condensation formed larger oligoamines. Hölttä (7) investigated the oxidation of spermidine to putrescine and 3-aminopropionaldehyde and of spermine to spermidine and 3-aminopropionaldehyde by polyamine oxidase from rat liver. Smith et al. (13) reported that the polyamine oxidase from Gramineae (Hordeum, Avena, Zea) oxidizes the polyamines spermidine and spermine at the secondary amino groups to give cyclic products derived from aldehydes: the product of spermine oxidation occurs as diazabicyclononane, while the product of spermidine oxidation is 1-pyrroline; 1,3-diaminopropane and hydrogen peroxide are formed in the case of both substrates. The oxidation of putrescine (12) by pea seedling amine oxidase yields 1-

pyrroline, and spermidine gives diazabicyclononane; the exact nature of the reaction with spermine was unclear. In this paper we report the oxidation of spermine, compared with putrescine and spermidine, by LSAO1. The spermine is oxidized at the terminal amino groups to a dialdehyde: 2 mol of H$_2$O$_2$ and 2 mol of NH$_4^+$ per mol of spermine are formed.

MATERIALS AND METHODS

Chemicals

Putrescine dihydrochloride, spermidine trihydrochloride, and spermine tetrahydrochloride were from Fluka, and recrystallized from ethanol. All other chemicals were obtained as pure commercial products and used without further purification: 2-aminobenzaldehyde, 1,3-diaminopropane dihydrochloride, and 3-methyl-2-benzothiazolone hydrazone from Sigma; acrolein from Serva; peroxidase VI, 4-hydroxy-3-methoxy phenylacetic acid and 2,4-dinitrophenylhydrazine from Sigma; thiosemicarbazide from Aldrich. LSAO was purified to electrophoretic homogeneity according to Floris et al. (5). Putrescine, spermidine, spermine, and 1,3-diaminopropane were chromatographed by TLC in phenol-H$_2$O (5:1 v/v) and cyclohexane-ethyl acetate (5:4 v/v) and visualized by a 0.1% ninhydrin spray in 90% 1-propanol.

Reaction Stoichiometry

Stoichiometry was determined as follows: (a) oxygen uptake was determined polarographically at 37°C by a Gilson Oxygen Graph equipped with a Clark electrode. The standard reaction mixture (1 mL) contained 50 μg catalase and the enzyme in 100 mM Ti-HCl buffer (pH 7.9). The reaction was started by addition of substrate solution after at least 10 min pre-incubation; (b) hydrogen peroxide was determined with the peroxidase: 4-hydroxy-3-methoxy phenylacetic acid method (9); (c) ammonia production was determined by the reaction with glutamate dehydrogenase (4); (d) l-pyrroline formed was estimated by 2-aminobenzaldehyde (6) as well as the ninhydrin method (10); (e) the monoaldehyde and dialdehyde production was determined by 3-methyl-2-benzothiazoline hydrazone (2, 11). 3-Aminopropionaldehyde was estimated as a thiosemicarbazone derivative according to Hölttä (7). Acrolein was estimated by fluorimetric determination according to Alarcon (1).

---

1 Abbreviation: LSAO, lentil seedling amine oxidase.
Spectroscopic Measurements

Absorption spectra of the enzyme were taken with a Cary model 219 spectrophotometer using a 1-cm lightpath cell. 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. Fluorescence spectra were obtained by a Perkin Elmer LS-3 spectrofluorimeter.

RESULTS

Reaction of Native LSAO with Spermine

The absorption spectrum of LSAO, in the visible range, shows a band centered at 498 nm. The addition of spermine (50 nmol) to 1 mL of the solution containing 10 nmol of LSAO in the absence of air, is followed by the disappearance of the 498 nm absorption peak and by the appearance of new absorption bands centered at 460, 430, and 350 nm as shown in the case of other substrates (putrescine) which was closely related to the modification of the enzyme with substrate (3). Oxygenation restores the pink-red color to its original intensity.

Rate of Oxidation of the Di- and Polyamines

Determined at their respective pH optima, initial activities with putrescine (0.1 M potassium phosphate [pH 7]), spermidine (0.1 M HCl [pH 7.5]), and spermine (0.1 M HCl [pH 7.9]) were in the ratio 100:42:20. The K_m values, obtained from double reciprocal plots, were 2.3 · 10^{-4}, 8 · 10^{-4}, and 4.4 · 10^{-4} M, respectively.

Oxygen Uptake

With 30 and 60 nmol of substrates, oxygen uptake for spermine was found to be twice that for putrescine and spermidine. With a larger amounts of spermine (10 μmol) the oxygen uptake decreased rapidly, stopped in spite of the large amount of substrate and oxygen remaining, and the enzyme became inactive. This curve for O_2 uptake was reproducible when the same amount of the enzyme was added at this point, and the amount of O_2 consumed is proportional to the amount of the enzyme; addition of spermine or putrescine, however, does not lead to resumption of O_2 uptake. On the average, each enzyme molecule turns over ten times before it becomes inactive, assuming a M of 150,000. These results show that spermine is a substrate and is also a reversible inhibitor. In fact, when the LSAO was dialyzed 18 h against 5 L of 0.1 M potassium phosphate buffer (pH 7.0), the inhibition was lost. An explanation for the loss of activity is that the enzyme is inactivated by the reaction products (see “Discussion”).

Hydrogen Peroxide Determination

Table I reported the stoichiometry obtained with 100 nmol of substrates in the oxidation by LSAO. We obtained 1.94, 0.97, and 0.9 nmol of H_2O_2 per nmole of spermine, spermidine, and putrescine, respectively.

<table>
<thead>
<tr>
<th>Substrate (100 nmol)</th>
<th>H_2O_2</th>
<th>NH_4^+</th>
<th>Aldehyde (10^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>97</td>
<td>89</td>
<td>5.1</td>
</tr>
<tr>
<td>Spermidine</td>
<td>90</td>
<td>88</td>
<td>4.7</td>
</tr>
<tr>
<td>Spermine</td>
<td>194 (sd ± 7.2)</td>
<td>190 (sd ± 7.6)</td>
<td>9.6</td>
</tr>
</tbody>
</table>

Ammonia Determination

NH_4^+ release was determined from the amount of NADH consumed in the presence of glutamate dehydrogenase and α-ketoglutarate. As shown in Table I, 1.9, 0.89 and 0.88 nmols of NH_4^+ were released per nmol of spermine, putrescine, and spermidine, respectively.

Aldehyde Determination

The aldehydes formed were assayed by the 3-methyl-2-benzothiazolone hydrazone. The molar absorbancy at 660 nm, obtained after the oxidation of spermine, was 9.6 · 10^3, twice that for oxidized putrescine and spermidine (molar absorbancy 5.1 and 4.7, respectively). Similar results were obtained by Bachrach and Reehes (2) in the oxidation of spermine and spermidine by beef serum amine oxidase. This observation is consistent with the assumption that spermine may be oxidized to a dialdehyde. The dialdehyde does not react with 2-aminobenzaldehyde or ninhydrin (10), indicating that it does not occur in aqueous solution in the monocyclic pyrorrulinium form, such as the aminoaldehyde originating from putrescine or spermidine (13). The formation of carbonyl compounds was also shown qualitatively by a reaction between 1 mL incubation mixture (100 nmol oxidized spermine) and 2,4-dinitrophenylhydrazine (0.2%) in 6 n HCl for 10 min at 37°C, followed by 2 mL of 3 n NaOH.

Other Products

When the LSAO reacts with 10 μmol of spermine, the enzyme became inactive (see “Oxygen Uptake”). After several additions of the same amounts of LSAO to the incubation, all the spermine (determined by TLC) disappeared from the incubation mixture while a new compound became evident. This compound is yellow-orange, with two absorption bands in the visible region centered around 520 and 430 nm: in 0.1 M HCl (pH 7.9), the A_{530}/A_{430} nm ratio is 0.5. The compound shows a fluorescens, with emission spectra at 460 and 570 nm when excited at 430 and 520 nm, respectively (Fig. 1, A and B). The compound reacts with ninhydrin and also with 2,4-dinitrophenylhydrazine, as would be expected for a
Spermine oxidation

Figure 1. Fluorescence spectra of the oxidation products of spermine. After ultrafiltration and centrifugation at 9000 rpm of the reaction mixture, the supernatant was lyophilized. Emission spectra were obtained by excitation at the maxima and excitation spectra by monitoring the emission at 460 (A) and 570 (B) nm (0.1 mg of lyophilized product in 1 mL 0.1 M Ti-HCl [pH 7.9]).

compound with free NH₂ groups and with carbonyl functions. Probably the dialdehyde obtained by the oxidation of spermine reacts with spermine (Schiff bases) or with dialdehydes (aldol condensation) and gives rise to a polymer containing carbonyl functions and free NH₂ groups. The nature of the condensation reaction is not known and is currently under investigation.

DISCUSSION

Data reported in this paper show that spermine is oxidized by lentil amine oxidase in the NH₂ terminal positions with the formation of a dialdehyde. The dialdehyde does not occur in aqueous solution in the monocyclic pyrroline form or in 1,5-diazabicyclononane, as reported for the oxidation of putrescine or spermidine (13). If the oxidation occurred at the secondary amino groups (7, 13) a variety of other compounds would be possible, such as 3-aminopropionaldehyde, 1,3-diaminopropane, putrescine (oxidized by the same LSAO to 1-pyrrroline), spermidine (oxidized by the same LSAO to 1-[3-aminopropyl]pyrroline) (12). If the oxidation occurred at the terminal amino groups, but the dialdehyde break down to putrescine (12), other compounds would be possible, such as acrolein (1) or 1-pyrrroline. Attempts were made to search these compounds (1,3-diaminopropane, 3-aminopropionaldehyde, acrolein, 1-pyrrroline, and diazabicyclononane) but without success. Oxygen uptake is 2 mol per mol of spermine; 2 mol of H₂O₂ and 2 mol of NH₄⁺ per mol of spermine are also formed. From the results obtained the oxidation of spermine may be presented by the following equation:

Spermine + 2 O₂ + 2 H₂O → dialdehyde
+ 2 NH₄⁺ + 2 H₂O₂.

The dialdehyde formed during the oxidation of spermine, highly reactive, may combine with the free amino groups of LSAO forming Schiff bases, resulting in enzyme inhibition. The enzyme molecule turns over ten times before it becomes inactive.

LITERATURE CITED