DEGRADATION OF LYSOPINE BY AN INDUCIBLE MEMBRANE-BOUND OXIDASE IN *AGROBACTERIUM TUMEFACIENS**

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Received 18 October 1972

1. Introduction

D-lysopine (*N*-α-propionyl-L-lysine) and D-octopine (*N*-α-propionyl-L-arginine) are non-protein amino acids simultaneously found in crown-gall tumors [1, 2]. As only those strains of *Agrobacterium tumefaciens* (the crown-gall tumor inducing bacteria) which were able to metabolize octopine were able to induce octopine containing tumors, Petit et al. [3] proposed that these characteristics might be evidence for genetic information transfer (at the tumor initiation stage) from the bacteria to the plant host-cell. Similar results were found for lysopine. In two previous reports [4, 5] we described the inducible lysopine metabolism by *A. tumefaciens*. This paper presents certain properties of the membrane-bound enzyme complex responsible for the oxidation of lysopine which leads to the formation of pyruvate and lysine: the oxidase activity is probably cytochrome-linked, does not require any cofactor and thus compares well with other amino acid oxidases found in *Pseudomonodacae* [6, 7]. This activity seems so far specifically induced by lysopine.

2. Material and methods

2.1. *Preparation of enzyme*

Culture conditions of *A. tumefaciens* (strain B_6) on a synthetic medium [8] containing lysopine (0.75 g/l) as a nitrogen source have been described [4]. The 105 000 *g* membranous pellet obtained from sonicated bacteria [5] constituted the routine source of enzyme and was kept frozen until use; it was then resuspended in a 0.04 M phosphate buffer, pH 7.6, containing 10^{-3} M lysopine; for colorimetric assays, the suspension was diluted to 1 to 2 mg protein/ml.

In some experiments preparations were obtained from bacteria grown in the same medium containing, instead of lysopine, NH_4Cl (0.3 g/l) or D-alanine (0.6 g/l) as a nitrogen source.

2.2. *Assays of lysopine oxidase*

The resuspended pellets converted lysopine into lysine with oxygen absorption. O_2 measurements were made by the conventional Warburg method at 25°. The flasks contained approx. 2 mg of protein, 60 μmoles of lysopine in the side arm and 0.1 ml of 10% KOH in the center well; the final volume of the mixture (pH 7.6) was 1 ml; lysine formed was measured at the end of the reaction by a densitometric method [5]. For each experiment blanks were made without lysopine, and the O_2 and lysine values were subtracted from those of the sample vessel. In some experiments phenazine methosulfate (PMS) (10^{-3} M), catalase (5000 units/ml) and ethanol (0.025 M), or semicarbazide (0.015 M) were added.

For routine assays a colorimetric method, using 2,6-dichlorophenol-indophenol (DCIP) as electron acceptor, was developed. Composition of a typical mixture appears in fig. 1. This method was also used to test the inducibility and substrate specificity of lysopine oxidase. Like lysopine, all substrates were tested at 0.02 M. No DCIP reduction occurred unless oxygen exclusion was first accomplished by N_2 flushin

* This work is part of a Doctoral Thesis (Doctorat ès Sciences) to be submitted.

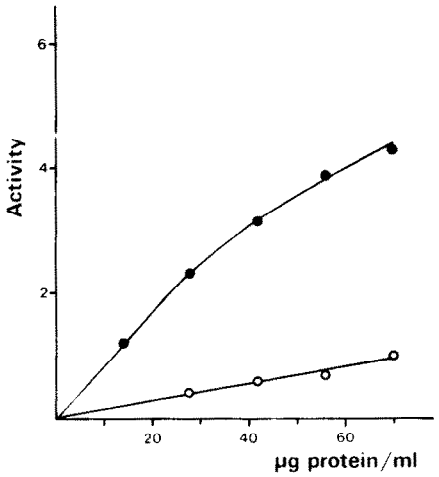

Fig. 1. Activity of lysopine-oxidase was assayed with (●—●—●) and without PMS (○—○—○). Assay mixtures contained: lysopine (2×10^{-2} M), KCN (1.5×10^{-2} M), DCIP (0.9×10^{-4} M). All compounds were dissolved in 0.2 M phosphate buffer, pH 7.6. Final volume: 0.45 ml. The blank contained all ingredients except lysopine. The activity is expressed in nmoles of DCIP reduced per min.

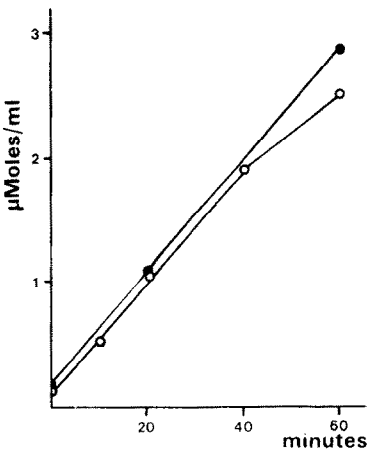

Fig. 2. Pyruvate and lysine formation from lysopine. Experimental procedures for pyruvate (○—○—○) and lysine (●—●—●) assays are described in sect. 2.3.

or unless KCN was added into the cuvette. The assay was performed in microcuvettes (0.5 ml) and the reaction started by the addition of DCIP. No reduction of the dye occurred without lysopine. Rates of reaction for lysopine, three or four times higher than those in the manometric method, were worked out using a molar absorbancy of 2.1×10^4 $cm^2 \times mole^{-1}$ for DCIP; absorbancy changes were proportional to the enzyme concentration up to changes of 0.1 per min. As PMS increased the reduction rate about 6-fold, it was thus routinely added (final conc. 5×10^{-5} M) (fig. 1).

2.3. *Other methods*

Lysine was determined by a densitometric method after paper electrophoresis of the samples and development with ninhydrin [5]; its optical form was controlled using lysine decarboxylase [9]. Pyruvate was detected and measured using the Sigma pyruvic acid test-kit no. 726 (LDH assay). D-lysopine was synthetized in the laboratory [10]; all other chemicals were commercial preparations (Sigma). Protein was determined by the method of Lowry et al. [11].

Direct and difference spectra were recorded on a Cary 15 spectrophotometer.

3. Results

3.1. *Products and stoichiometry of the reaction*

In a mixture containing the resuspended pellet and lysopine, this amino acid is converted into lysine under aerobic conditions [5]. The reaction, as measured by O_2 uptake, is inhibited by addition of 5×10^{-3} M KCN. According to the O_2 uptake, rates of reaction range from 2 to 3 nmoles O_2 absorbed/min/mg of protein. We checked with lysine decarboxylase that the lysine formed was L-lysine. Pyruvate formation, evidenced by its reactivity with lactate dehydrogenase, occurs simultaneously; no pyruvate formation was observed when assays were run without lysopine. Kinetics of lysine and pyruvate formation (fig. 2) show that the molecular ratio is 1.

With or without PMS the molecular ratio lysine/O_2 was found to be 2 (table 1). O_2 composition was not due to pyruvate oxidation, since the addition of semicarbazide to the reaction mixture as a trapping agent did not change the O_2 absorption rates. No changes of the ratio occurred either when catalase and ethanol were added into the flasks as peroxide trapping agents [12]. Moreover no catalase activity was found in the resuspended pellet. The fact that the reaction is inhibited by cyanide, and that there is no peroxide formation, suggests that the reaction might be cytochrome linked. Examination of the spectral changes during incubation of the particulate extract with

Table 1
Stoichiometry of O_2 consumption and lysine formation.

Addition	O_2 (micromoles/vessel)	Lysine (micromoles/vessel)	$\frac{Lys}{O_2}$ ratio
None	0.69	1.25	1.8
None	0.91	1.79	1.9
Catalase + ethanol	1.01	1.91	1.8
PMS	2.01	1.91	1.8
PMS	2.16	2.18	1.8
PMS, catalase + ethanol	2.09	4.38	2.0
PMS, semicarbazide	0.71	1.55	2.1

Experimental conditions as described in the text under sect. 2.2. Incubation time 180 min.

lysopine actually showed reduction of cytochrome *c*- and *b*-like materials.

3.2. *Inducibility and substrate specificity of lysopine oxidase*

Inducibility and specificity of the oxidase were assayed with the DCIP test (table 2). Lysopine oxidase activity was only found, as previously noted [5], in pellets from lysopine-grown bacteria. Although all D-amino acids tested but D-glutamate were oxidized by the pellets, their individual oxidation rates were not so much affected by the growth conditions as lysopine oxidation rate is.

Table 3
Inhibitory effect of L-lysine and L-arginine on particulate extracts of lysopine grown cells.

	Rate*	Inhibition (%)
Lysopine	61	0
Lysopine + lysine (0.02 M)	54.6	12
Lysopine + lysine (0.04 M)	45.6	38
Lysopine + arginine (0.02 M)	49	20
Lysopine + arginine (0.04 M)	49	20

* nmoles DCIP reduced/min/mg protein.

Octopine was oxidized only by preparations from induced cells and at the same rate as lysopine. As these amino acids have an analog structure we wondered whether L-lysine or L-arginine would compete for a binding site on the enzyme; inhibitory effects were actually recorded (table 3).

4. Discussion

Particulate amino acid oxidases found in *Pseudomonas* [6, 7, 13] were shown to consist of a dehydrogenase moiety linked with cytochromes. Although these enzymes have been also shown to contain covalently-bound FAD, they do not produce hydrogen peroxide during reaction, being therefore different from classical mammalian D-amino acid oxidases.

Our results are consistent with such a pattern: lysopine oxidation is probably cytochrome linked, we showed [5] that neither NAD nor FMN had

Table 2
Inducibility and substrate specificity of lysopine oxidase.

	NH_4Cl grown cells		D-alanine grown cells		Lysopine grown cells	
D-Lysopine	1.2	(1.8)	0.9	(1)	95	(132)
D-Octopine	1.2	(1.8)	0.9	(1)	95	(132)
D-Alanine	63.3	(100)	86	(100)	72	(100)
D-Valine	23	(35)	22.3	(25.9)	55	(76.9)
D-Phenylalanine	19.2	(29.6)	53	(61.6)	55	(76.9)
D-Histidine	16.2	(25)	27.4	(31.8)	30	(41.6)
D-Serine	11.5	(18)	17.4	(31.8)	25	(34.7)
D-Glutamate					0.9	(1.2)

All substrates were tested at 0.02 M. The rates are expressed in nmoles of DCIP reduced/min/mg protein; numbers in parentheses represent the percent of activity by comparison with D-alanine.

stimulatory effects; no hydrogen peroxide was formed during the reaction. According to these data, the pathway of lysopine degradation might be:

$$\text{D-Lysopine} \longrightarrow 2e^- + 2H^+ + (X)$$
$$(X) + H_2O \longrightarrow \text{L-lysine} + \text{pyruvate}$$

(X) being the Schiff base formed by lysine and pyruvate: electron transport through the cytochrome system would account for the oxygen absorption.

The elucidation of the role of PMS, the structure of the enzymatic complex and its specificity towards several substrates must await further purification of the enzyme.

Induction of lysopine oxidase seems to be closely related to the presence of lysopine in the growth medium: although the reaction takes place on the $[CH_3-CH-COOH]$ part of the lysopine molecule, D-alanine alone is not an inducer. This might be in agreement with the hypothesis of Petit et al. [3]. Nevertheless we have no evidence yet as to the absolute necessity of the lysine moiety of the lysopine molecule for the induction. The induction specificity might be only due to the presence of the $\geqslant C-NH-C\leqslant$ part of the molecule.

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