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CYCLOPENIN *M*-HYDROXYLASE—AN ENZYME OF ALKALOID METABOLISM IN *PENICILLIUM CYCLOPIUM*

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Key Word Index—*Penicillium cyclopium*; Ascomycetes; fungi; developmental phases; benzodiazepine alkaloids; biosynthesis; cyclopenin *m*-hydroxylase; mixed function oxygenase.

Abstract—Cyclopenin *m*-hydroxylase transforms cyclopenin, one of the two major alkaloids of *Penicillium cyclopium*, into cyclopenol. The enzyme belongs to the group of mixed function oxygenases. It needs molecular oxygen and a hydrogen donor (NAD(P)H, ascorbic acid, tetrahydropteridine) as cosubstrates, and it is inhibited by CN^- and SCN^- but not by CO. Inhibition by dicoumarol indicates that it may be a flavoprotein. With the exception of the alkaloid viridicatin, all tested compounds structurally related to cyclopenin are hydroxylated. The hydroxylase activity is measurable in the cultures at the end of the growth phase, i.e. during the period of alkaloid metabolism.

INTRODUCTION

The development of emerged cultures of *P. cyclopium* proceeds in 3 stages: the germination phase, the growth phase of the hyphae (trophophase) and the idiophase. In the idiophase cell specialization takes place, including the formation of the conidiospores and alkaloid metabolism is very active [1]. One of the last reactions of alkaloid biosynthesis is the *m*-hydroxylation of cyclopenin [2] (Fig. 1). *In vivo* experiments (incorporation of oxygen from $^{18}O_2$ into the hydroxy group accompanied by an NIH-shift) [3] have shown that the hydroxylation is catalysed by a mixed function oxygenase (cyclopenin *m*-hydroxylase). The present paper describes the *in vitro* measurement of this enzyme and its partial characterization.

RESULTS AND DISCUSSION

Idiophase cultures of *P. cyclopium* were used in the following experiments. These cultures form cyclopenol in the hyphae and in the conidiospores. However, in the conidia high activities of cyclopenase are present [4]. Cyclopenase transforms cyclopenin and cyclopenol to viridicatin and viridicatol, respectively and therefore competes with the *m*-hydroxylase for its substrate cyclopenin. Grinding briefly with sand was most suitable in disintegrating the hyphae without disruption of the conidiospores. Only a small amount of cyclopenase activity was set free during this treatment whereas cyclopenin *m*-hydroxylase activity in the homogenate was comparatively high. The enzyme activity measurable

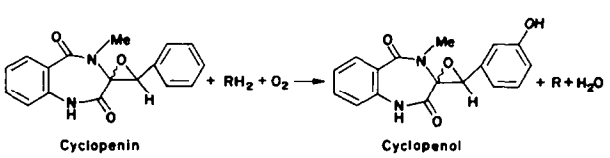


Fig. 1. Reaction catalysed by cyclopenin *m*-hydroxylase (R = hydrogen donor).

(7·5 nkat/cm² culture area) is nearly twice that calculated from the cyclopenol production of the cultures *in vivo* (4·3 nkat/cm² culture area). Similar results were found for cyclopeptine dehydrogenase, an enzyme involved in cyclopenin biosynthesis [5]. In both cases the absence of V_{max} conditions may be the reason for the lower enzyme activity in the living cells. However the greatly reduced cyclopenase activity of the intact conidiospores is due to separation of the enzyme and its substrates by compartmentation [6].

Cyclopenin *m*-hydroxylase, like most other mixed function oxygenases, needs a hydrogen donor as cosubstrate. NADH, NADPH, ascorbic acid, and D,L-6-methyltetrahydropteridine may be used (Table 1). Combination of these substances does not increase the activity of the enzyme. Similar results were found for instance with *p*-coumaric acid hydroxylase [7] and the other mixed function oxygenase of the alkaloid metabolism of *P. cyclopium*, dehydrocyclopeptine epoxidase [8].

Addition of α-ketoglutaric acid (0·33 mM) (cf. [9, 10]) to ascorbic acid containing incubation mixtures reduces the speed of hydroxylation. Cyclopenin hydroxylase thus belongs to that group of mixed function oxygenases which as for instance dopamine β-monooxygenase (E.C. 1.14.17.1) and *p*-coumarate 3-monooxygenase (E.C. 1.14.17.2) use ascorbate as a direct hydrogen donor (cf. [10]). The parallel dehydrogenation of NADH and NADPH was shown to be a property of the hydroxylase itself. It is not due to the presence of NAD(P) transhydrogenase (E.C. 1.6.1.1) in the enzyme preparations [8].

Most of the *m*-hydroxylase activity of sand homogenates is unspecifically bound to particles from which it can be solubilized by surface active agents (e.g. sodium deoxycholate). For a partial purification of the solubilized enzyme ammonium sulphate precipitation and calcium phosphate gel treatment was found to be appropriate. By a combination of both methods an increase of the sp act by a factor of about 50 was found. However, further purification was difficult due to the rapid loss

Table 1. Cosubstrates of cyclopenin *m*-hydroxylase†

No.	Cosubstrates (mM) and enzymes (nkat per test)	Relative enzyme activity
1	None	0
2	NAD^+ 0·3, EtOH 670, alcohol dehydrogenase 800	100
3	2 + NADH 0·2	120
4	$NADP^-$ 0·3, Glc 6-phosphate 2 Glc 6-phosphate dehydrogenase 100	110
5	4 + NADPH 0·2	130
6	4 + nicotinamide 50	160
7	Ascorbate 0·66*	100
8	D,L-6-Methyl-5,6,7,8-tetrahydro-pteridine 0·66	95

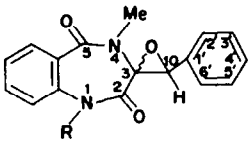
* High ascorbic acid concentrations decrease the hydroxylase activity (cf. [21]). †0·2 nkat of a roughly purified enzyme preparation (20–40% $(NH_4)_2SO_4$ saturation) were used per test.

of enzymatic activity (half life time at 0° *ca* 16 hr; at 25°, 30–40 min). By treatment with calcium phosphate gel the absolute enzyme activity is increased more than two fold. A similar effect was found after purification of crude extracts by Sephadex G 25 or during solubilization of the particle bound enzyme by sodium deoxycholate. By all these methods tightly bound phenolic compounds are removed from the hydroxylase containing protein fractions.

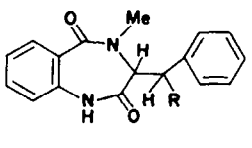
It is of interest that a certain amount of viridicatol formed for instance by cyclopenase present in the *m*-hydroxylase preparations binds to the proteins by hydrogen bridges. Probably it is the *m*-hydroxylase itself which interacts with the viridicatol. Thus, the binding capacity of cyclopenin *m*-hydroxylase enriched protein fractions is especially high. In contrast serum albumin is inactive. After addition of urea the bound viridicatol is liberated. In routine tests therefore MeOH (viridicatol in aqueous solutions is extremely insoluble) and urea (for liberation of viridicatol from proteins) were added after incubation.

Both cyclopenol and viridicatol, due to the phenolic group present in the molecule, give a positive Emmerson reaction which can be used for quantitative determinations. The orange–red products formed in contrast to those of many other phenols, are soluble in apolar solvents. Both have nearly the same molar extinction. Addi-

Table 2. Hydroxylation of compounds related to cyclopenin



(1)



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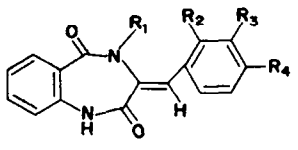

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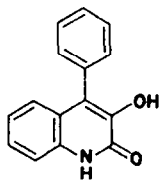

(4)

Compound	Structure	Relative rate of hydroxylation
Cyclopenin	I (R=H)	100
1-Methylcyclopenin	I (R=Me)	72
Cyclopeptine	II (R=H)	22
10-Hydroxy cyclopeptine	II (R=OH)	60
Dehydrocyclopeptine	III (R_1,R_2,R_3,R_4=H)	28
2′,4′-Dichlorodehydrocyclopeptine	III (R_1,R_3=H; R_2,R_4=Cl)	28
4′-Chlorodehydrocyclopeptine	III (R_1,R_2,R_3=H; R_4=Cl)	35
3′-Methoxydehydrocyclopeptine	III (R_1,R_2,R_4=H; R_3=OMe)	35
4-N-Benzyldehydrocyclopeptine	III (R_1=CH_2–C_6H_5; R_2,R_3,R_4=H)	33
Viridicatin	IV	0

Experimental conditions as given in the experimental part and Table 1. 3·4 μmol of the substrates in 0·1 ml MeOH were added per test.

Table 3. Effect of inhibitors on cyclopenin *m*-hydroxylase activity†

Compound added (mM)	Relative enzyme activity	Compound added (mM)	Relative enzyme activity
None	100	$FeSO_4$ 15	100
Dicoumarol 5	60	$CuSO_4$ 15	0
KCN 10	15	CuCl 15	0
KSCN 10	10	$MgCl_2$ 15	100
p-Chloromercuri-benzoate 5	15	KCl 15	100
o-Phenanthroline 5	100	$MnCl_2$ 15	66
EDTA 5	100	$ZnCl_2$ 15	80
O_2:N_2 20:80	100*	O_2:CO 20:80	230*

* Instead of shaking a stream of the gas mixture was passed through the test solution.
† Experimental conditions as given in the experimental part and Table 1.

tion of Fe^{2+}-ions reduces the extinction of the blanks probably due to complex formation with some of the phenols present. Determination of cyclopenol–viridicatol, however, was not influenced.

By cyclopenin *m*-hydroxylase preparations all tested benzodiazepines were transformed into hydroxyderivatives (Table 2). Cyclopenin hydroxylation had the highest velocity. The quinoline derivative viridicatin was not attacked. In spite of the *in vitro* hydroxylation of cyclopeptine and dehydrocyclopeptine in the living cell hydroxy derivatives of these intermediates of cyclopenin biosynthesis (due to compartmentation?) were never found.

Cyclopenin *m*-hydroxylase is not inhibited by CO (Table 3). This may indicate that the enzyme does not belong to the group of mixed function oxygenases which contain a cytochrome P-450 moiety (however, cf. [11]). The increase of enzyme activity in the presence of CO indicates that in the absence of this compound, competition for hydrogen donors with CO sensitive enzymes occurs. Addition of cytochrome C (10 μM) to incubation mixtures reduces the measurable *m*-hydroxylase activity probably by activating these CO sensitive enzymes.

The decrease of enzyme activity after addition of dicoumarol, an inhibitor of flavin enzymes [12], indicates that the *m*-hydroxylase belongs to this group of proteins. KCN and KCNS inhibit the enzyme activity, too. The hydroxylase thus may be a metallo-protein. However, addition of FAD as well as of different metal ions (Table 3) did not increase the enzyme activity.

Figure 2 shows that at the beginning of the idiophase, i.e. after cessation of mycelial growth, both cyclopenol excretion and cyclopeptine *m*-hydroxylase activity became measurable. Both processes have a maximum on the 6th day after inoculation. This behaviour resembles that of the other *in vitro* measurable enzymes of cyclopenin-cyclopenol biosynthesis, cyclopeptine dehydrogenase and dehydrocyclopeptine epoxidase under the same culture conditions [8]. Detailed quantitative investigations of the alkaloid production *in vivo* in the presence [13] and absence [14] of inhibitors of protein synthesis indicate that cyclopenin *m*-hydroxylase as well as the other enzymes of cyclopenin–cyclopenol biosynthesis are synthesized *de novo* during the idiophase.

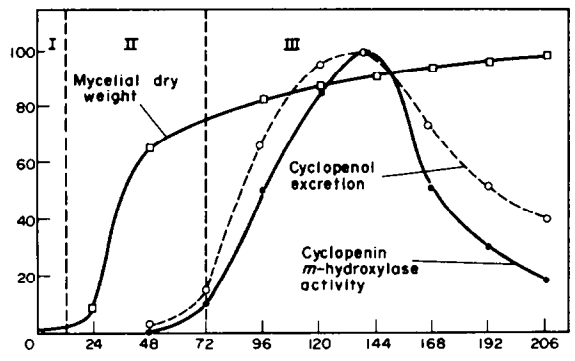

Fig. 2. Phase dependence of cyclopenin *m*-hydroxylase expression during the development of emerged cultures of *Penicillium cyclopium*. Cultivation of the mould by 24 hr replacement of the nutrient soln by NL II, separation of the mycelium (hyphae) from the conidiospores, determination of dry wt, of protein, and of excreted cyclopenol were performed as described by [1]. I Germination phase; II Trophophase; III Idiophase: □——□ Mycelial dry wt (100 = 3·7 mg/cm² culture area); ●——● cyclopenin *m*-hydroxylase activity in the sand homogenate (100 = 7·5 nkat/cm² culture area); O-----O rate of cyclopenol excretion (100 = 5 μg/cm² culture area/hr).

EXPERIMENTAL

Cultivation. P. cyclopium Westling strain SM 72 [15] used in these investigations was cultivated by the batch method according to [16].

Chemicals. Cyclopenin, cyclopenol, viridicatin and viridicatol were prepared according to [17]. 1-Methylcyclopenin was prepared from cyclopenin [18]. The other compounds listed in Table 3 were synthesized according to [19].

Disintegration of mycelial cells. 1 part of the culture (age 5–6 days) was mixed with 2 parts of sand and ground in a mortar for 10 min. After addition of 2 parts 66 mM Pi buffer pH 7·4 containing 0·5% Na deoxycholate and 0·05% 2-mercaptoethanol the grinding was continued for another 20 min. The homogenate was centrifuged 30 min at 2500 *g*.

Purification of cyclopenin m-*hydroxylase.* By $(NH_4)_2SO_4$-precipitation the highest enzyme activity was found in the fraction from 20–40% saturation (enrichment 2·5 times, yield 35%). For Ca phosphate gel treatment this protein fraction was dissolved in 66 mM Pi buffer pH 6·5 and treated with the gel suspension [20] in a ratio of 1 mg dry wt/mg protein. After 1 hr the mixture was centrifuged and the sediment eluted successively with Pi buffer pH 6·6, 0·33 M and 0·66 M (enrichment of the enzyme in the eluate 20 times, yield 235%).

Determination of cyclopenin m-*hydroxylase activity.* In routine tests the mixture of cyclopenin 3·4 μmol in 0·1 ml MeOH, NADP 0·45 μmol, glucose-6-phosphate 2·8 μmol, glucose-6-phosphate dehydrogenase 100 μkat and the enzyme prepn (1 ml in 66 mM Pi buffer pH 6·5 total vol 1·6 ml) were shaken 30 min at 25°. After the incubation 1·3 ml MeOH and 1 ml 6 M urea soln were added. Determination of the *m*-hydroxylated compounds was performed according to [1].

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