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DEHYDROCYCLOPEPTINE EPOXIDASE FROM *PENICILLIUM CYCLOPIUM*

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Abstract—Dehydrocyclopeptine epoxidase (DE) activity was determined in cell free preparations of *Penicillium cyclopium*. The enzyme transforms dehydrocyclopeptine into cyclopenin by a mixed function oxygenation. It needs molecular oxygen and uses NAD(P)H, ascorbate or D,L-6-methyl-5,6,7,8-tetrahydropteridine as cosubstrates. DE is inhibited by CN^-, SCN^-, 1,10-phenanthroline, EDTA, 2,2'-bipyridine, sodium diethyldithiocarbamate, dicoumarol, *p*-chloromercuribenzoate and ions of different heavy metals, but not by CO and the lead salt of diethyldithiocarbamate. These properties indicate a specific importance of Fe^{2+}-ions, SH-groups and flavins. DE activity is increased by Fe^{2+} and FAD. The enzyme may be therefore a Fe^{2+} activated FAD containing flavoprotein. DE was enriched 268-fold by $(NH_4)_2SO_4$ precipitation and chromatography on Sephadex G-200. Its MW estimated by Sephadex chromatography, exceeds 480000.

INTRODUCTION

Feeding of radioactive labelled compounds to emerged cultures of *Penicillium cyclopium* Westling has shown that the benzodiazepine alkaloid dehydrocyclopeptine is an intermediate in the biosynthesis of the corresponding epoxides cyclopenin and cyclopenol [1]. By incorporation of ^{18}O from $^{18}O_2$ into the epoxide ring it was demonstrated that a mixed function oxygenase, dehydrocyclopeptine epoxidase (DE), catalyses the epoxidation [2]. This paper reports on this enzyme.

RESULTS AND DISCUSSION

Preliminary experiments indicated that DE activity in cell free preparations of *P. cyclopium* is very small. A sensitive method was therefore developed for the determination of small amounts of cyclopenin, the product of DE action, in the presence of a large quantity of the substrate dehydrocyclopeptine. This procedure is based on the following principles (cf. Scheme 1):

(1) Radioactive labelling of the substrate. Dehydrocyclopeptine-[G-^{3}H] was prepared from Wilzbach labelled 10-hydroxycyclopeptine-[G-^{3}H] via the 10-acetoxy derivative [3]. By direct tritiation of dehydrocyclopeptine no dehydrocyclopeptine-[G-^{3}H] was obtained, due to the reduction of the double bond.

(2) Transformation of the enzymatically produced cyclopenin-[G-^{3}H] into viridication-[G-^{3}H]. This reaction prevents hydroxylation of cyclopenin-[G-^{3}H] by cyclopenin *m*-hydroxylase [4] and simplifies separation of the enzymatic product from excess substrate. Viridicatin, in contrast to the benzodiazepine alkaloids (e.g. dehydrocyclopeptine and cyclopenin), is tightly bound to Al_2O_3 and TLC on this material does not move it from the origin, whereas dehydrocyclopeptine by use of sufficiently polar solvents migrates near the front. By repeated development even traces of dehydrocyclopeptine are removed from the starting point.

A large excess of the enzyme cyclopenase [5, 6] was used for catalysing the transformation of cyclopenin-[G-^{3}H] into viridicatin-[G-^{3}H]. Cyclopenase does not interfere with the epoxidase and thus was added directly to the enzymatic test.

(3) Oxidation of viridicatin-[G-^{3}H] to 2-aminobenzophenone-[G-^{3}H]. Unfortunately viridicatin bound to Al_2O_3 is difficult to elute and measurement of its radioactivity *in situ* has a low counting efficiency. Viridicatin-[G-^{3}H] was therefore oxidized to 2-aminobenzophenone-[G-^{3}H] [7], which is easily liberated and carries the total radioactivity of viridicatin-[G-^{3}H]. In the routine determination of radioactivity it was not necessary to separate the 2-aminobenzophenone from the oxidation mixture.

The method described was proved to be selective and suitable for quantitative work. In dependence on the specific radioactivity of the dehydrocyclopeptine, which is used as substrate, it is more than 100-times as sensitive as the photometric determination with Fe^{3+} of alkaloids of the cyclopenin-viridicatin group [8].

DE activity is measureable in homogenates of alkaloid-producing idiophase cultures of *P. cyclopium* after cell disintegration by grinding with sand or dry ice and by treatment with acetone or the X-press. Whereas by the former two methods hyphal cells are destroyed, only the latter methods also disintegrate the conidiospores [5]. Highest specific DE activities were found after acetone treatment (Table 1). Acetone homogenates are nearly free from low MW compounds, e.g. of the alkaloids cyclopenin and cyclopenol as well as viridicatin and viridicatol, which inhibit DE activity, of hydrogen donors and of effectors. They can be used, therefore, for determination of the influence of these substances on DE activity. However, in acetone preparations DE is relatively

Table 1. DE activity in cell preparations of *P. cyclopium*

Method of cell disintegration	Dehydrocyclopeptine epoxidase activity				
	pkat/g fr. wt		pkat/mg protein		
	Homogenate	Supernatant (1400 *g*/20 min)	Homogenate	Supernatant (1400 *g*/20 min)	% in the 'cell wall fraction' of DE activity of homogenates
Grinding with sand	1.00*	0.40	0.38*	0.20	56*
Grinding with dry ice	0.70*	0.16	0.33*	0.08	86*
Treatment with acetone	1.24	0.80	0.55	0.40	37
X-Press treatment	4.20	2.80	0.33	0.28	36

* Contains unbroken cells, e.g. conidiospores. Because these cells add to the measured protein content, DE activity/mg protein is too low.

unstable (loss of enzyme activity at 0° within 24 hr 100%, in presence of 1 mM Fe^{2+} 75%). DE activity is higher in sand homogenates (loss of activity at 0° within 24 hr 70%, in presence of 1 mM Fe^{2+} at pH 6.5 less than 10%). Sand homogenates can easily be prepared in large quantities and thus were used for preparative purposes and purification of the enzyme. A relatively large part of DE after cell disintegration is found in the easily sedimentable 'cell wall fraction' (Table 1). In this respect DE resembles cyclopeptine dehydrogenase [9] and cyclopenin *m*-hydroxylase [4], two other enzymes of the alkaloid metabolism of *P. cyclopium*. Most of the bound DE, however, can be solubilized by surface active agents (e.g. 1% deoxycholate).

DE shows the following basic properties: Maximum activity at pH 7.5 (0.1 M Tris–HCl), linearity of cyclopenin formation for 30 min on incubation at 35°, K_m of dehydrocyclopeptine 0.17 mM. It needs a hydrogen donor as cosubstrate. NADH (K_m = 0.14 mM), NADPH (K_m = 0.12 mM), ascorbic acid (optimum concentration 7.5 mM) and D,L-6-methyl-5,6,7,8-tetrahydropteridine (K_m = 3.14 mM) are used for this purpose (Table 2). Because no NAD(P)-transhydrogenase activity (EC 1.6.1.1.) is detectable in P. *cyclopium* the parallel use of NADH and NADPH is a property of DE itself.

DE, like several other mixed function oxygenases (cf. [10–13]), is able to use ascorbic acid and D,L-6-methyl-5,6,7,8-tetrahydropteridine directly as hydrogen donors. Addition to ascorbic acid containing tests of fumaric acid [14, 15] but not of α-ketoglutaric acid (cf. [16–19]) increases enzyme activity (Table 2). The high optimum concentration of ascorbic acid as well as the high K_m-value of D,L-6-methyltetrahydropteridine, which is much larger than the K_m-values of NADH and NADPH, indicate that ascorbate and the pteridine *in vivo* are not important cosubstrates, in spite of the fact that *in vitro* the highest enzyme activity is measureable with the latter compound. Combinations of NADH, NADPH and ascorbate gave no additive effects. However, addition of FAD, but not of FMN or riboflavin to tests containing NADH increases DE activity (Table 2). The increase of enzyme activity after addition of nicotinamide to tests containing

Table 2. Influence of hydrogen donors and effectors on DE activity

Additives (mM)	(A) Without addition of Fe^{2+} Composition of the test: Dehydrocyclopeptine 0.16 mM, Tris–HCl 60 mM DE* 0.5 pkat. Relative DE activity (%)	(B) With addition of Fe^{2+} Composition of the test: Dehydrocyclopeptine 0.16 mM, Tris–HCl 60 mM, $FeSO_4$ 5.0 mM DE* 0.5 pkat. Relative DE activity (%)
None	1	0
+ NADH 0.6	103	680
+ NADPH 0.6	100	690
+ Ascorbate 7.5†	100	690
+ Ascorbate 5.0 and α-ketoglutarate 2.5	97	682
+ Ascorbate 5.0 and fumarate 2.5	130	754
+ D,L-6-Methyl-5,6,7,8,-tetrahydropteridine 10	247	935
+ Folate 1.75 + NADH 0.6	110	—
+ Nicotinic acid amide 40 + NADH 0.6	130	845
+ Riboflavin 0.5 + NADH 0.6	100	—
+ FMN 0.5 + NADH 0.6	100	681
+ FAD 0.125 + NADH 0.6	144	729

* Prepared by Me_2CO disintegration and $(NH_4)_2SO_4$-precipitation (20–40%)
† Optimum concentration; more ascorbate decreases DE activity (cf. also [11]).

Table 3. Influence of metal ions and inhibitors on DE activity. Standard test: dehydrocyclopeptine 0.16 mM, NADH 0.6 mM, Tris-HCL 50 mM, DE prepared by Me_2CO disintegration and $(NH_4)_2SO_4$ precipitation (20–40%) 0.5 pkat

Added to the standard test	mM	Relative enzyme activity (%)	Added to the standard test	mM	Relative enzyme activity (%)	Added to the standard test	mM	Relative enzyme activity (%)
Non		100	KCN	2	33	Diethyldithio-		
Ca^{2+}	1	44		10	0	carbamate		
Co^{2+}	1	86	KSCN	2	35	sodium salt	0.1	80
Cu^{+}	1	64		10	0		0.2	70
Cu^{2+}	1	27	1,10-Phenan-	0.002	62		0.4	58
Fe^{2+}	1	172	throline	0.25	44		1.0	52
	5*	631		0.50	9	lead salt	0.1	100
Fe^{3+}	1	100	EDTA	0.02	42		0.2	100
Hg^{2+}	1	42		0.25	5		0.4	100
K^{+}	1	100		0.50	0		1.0	99
Mg^{2+}	1	100	2,2'-	0.5	87	Dicoumarol	0.005	20
Mn^{2+}	1	110	Bipyridine	1.0	67		0.02	2
Na^{+}	1	100		1.5	47		0.05	0
Ni^{2+}	1	41		2.0	36	*p*-Chloro-	0.02	73
Zn^{2+}	1	72				mercuri-	0.5	27
						benzoic acid	1.0	0
$O_2:N_2$ 80:20†		100				O_2:CO 80:20†	195	

* Optimum concentration; use of higher Fe^{2+} amounts decreases DE activity probably by unspecific interference with the enzyme protein.

† The gas mixtures were passed through the standard text (50 ml/min).

NADH (Table 2) cannot so far be explained. This was also found with other mixed function oxygenases, such as sterol demethylase which does not contain cytochrome P_{450} [20].

Fe^{2+}-ions which were found to stabilize DE (see above) also cause a marked increase of enzyme activity (Tables 2 and 3) (cf. also [18, 21–26]). However, they are not able to substitute for the electron donors used as cosubstrates (cf. Table 2). DE is inhibited by CN^{-}, CNS^{-}, 1,10-phenanthroline, EDTA, 2,2'-bipyridine and diethyldithiocarbamate sodium salt (Table 3), i.e. compounds chelating metal ions. Inhibition by 1,10-phenanthroline and 2,2'-bipyridine is specific for Fe^{2+}-ions, whereas sodium diethyldithiocarbamate, but not the lead salt, reacts with Fe^{2+} as well as Fe^{3+} (unpublished results, cf. also [27, 28]). These results indicate the specific importance of Fe^{2+} for DE activity. Dicoumarol, an inhibitor of flavin enzymes [29], even in small concentrations, reduces DE activity (Table 3). Thus DE, like cyclopenin *m*-hydroxylase [4], the other mixed function oxygenase of the alkaloid metabolism of *P. cyclopium*, may be a flavoprotein.

The lack of inhibition of DE activity by CO (Table 3) indicating that the enzyme does not contain cytochrome P_{450} ([20, 30, 31], however, cf. [32]), corresponds with this assumption. The activity increase after addition of CO which is also found with other mixed function oxygenases [4, 20, 33] cannot be explained.

p-Chloromercuribenzoic acid decreases the enzyme activity (Table 3). This inhibition corresponds with depression by Co^{2+}, $Cu^{+/2+}$, Hg^{2+}, Ni^{2+} and Zn^{2+}-ions indicating the necessity of SH-groups for catalytic action. Together with the increase in DE activity by means of FAD and Fe^{2+} the action of the tested inhibitors shows that DE is a Fe^{2+}-activated, FAD containing flavoprotein whose activity depends upon the presence of SH-groups.

DE was purified 268-fold by $(NH_4)_2SO_4$ precipitation and Sephadex chromatography (Table 4). The loss of specific enzyme activity after $(NH_4)_2SO_4$ treatment is due to inhibition by co-precipitated alkaloids which were present in the sand homogenate. By means of the following chromatographic purification, however, not only the

Table 4. Purification of DE

Preparation	DE activity pkat	Protein mg	Specific activity pkat/mg protein	Enrichment factor	Yield
Supernatant (20000 *g*/20 min) of sand homogenate	140	300	0.47	1	100
$(NH_4)_2SO_4$ precipitation 20–40%	40	90	0.45	0.96	29
Eluate from Sephadex G-200	8	0.064	1258	268	5.7

bulk of proteins but also these alkaloids are separated from DE. Thus the specific enzyme activity in the eluate is significantly increased. Chromatography on Sephadex G-200 of DE together with reference substances has shown that the enzyme has a MW of little more than 480 000.

EXPERIMENTAL

P. cyclopium strain SM 72 [34] was grown in batch cultures on NL I [8]. The cultures were harvested on the 6th day after inoculation.

Chemicals. Dehydrocyclopeptine-[G-^{3}H] prepared according to [3] was purified by TLC with System I (Si gel G, toluene–HOAc–EtOH–H_2O (87:8:2:0.2) development 3×). It was eluted with MeOH–EtOAc (1:1) and crystallized from EtOAc–pentane. For the removal of impurities with the same R_f value as that of viridicatin it was rechromatographed with system II (Al_2O_3 PF_{254}, MeOH–Me_2CO (1:1) development 3×). The eluted material was mixed with non-radioactive dehydrocyclopeptine to a sp. act. of 1.5 μCi/μmol. This mixture showed a single peak of radioactivity by chromatography in Systems I (R_f 0.55) and II (R_f 0.95) hiridicatin R_f 0.

Disintegration of pyphae by grinding with sand or dry ice. One part of the culture was mixed with 2 parts of sand or dry ice and ground for 10 min. After addition of 2 parts 0.1 M Tris–HCl buffer pH 7.5 the grinding was continued for another 20 min.

Disintegration of hyphae and conidiospores by Me_2CO. Cell material (10 g) and 20 g dry ice were ground. The homogenate was mixed first with 15 ml H_2O and then with 135 ml Me_2CO (−20°). After 5 min the insoluble material was removed by suction. Traces of Me_2CO were removed in a stream of air. After 1 hr the dry residue was mixed with 20 ml 0.1 M Tris–HCl buffer pH 7.5.

Disintegration of hyphae and conidiospores by the X-press. One part of the cell material and 2 parts of dry ice were ground for 10 min. The homogenate was mixed with 10 parts of 0.1 M Tris–HCl buffer pH 7.5. The suspension was pressed 5-times with the Biotec X-press (Bromma, Sweden) at −30° and allowed to thaw at 4°.

Determination of DE activity. The test mixture contained 0.6 mM NADH, 0.16 mM dehydrocyclopeptine-[G-^{3}H] (1.5 μCi/μmol), 50 mM Tris–HCl pH 7.5, 15 mg of Me_2CO treated conidiospores (as a crude cyclopenase preparation; 0.28 nkat/mg dry wt [5]) and 0.5 ml of the enzyme prepn containing *ca* 0.5 pkat DE; total vol. 2 ml. After 30 min incubation at 37° the enzyme reaction was stopped by shaking the mixture with 10 ml EtOAc. Na_2SO_4 (7.5 g) was added 10 min later to bind the aq. soln. 7 ml of the organic phase was concd in a stream of air and the viridicatin-[G ^{3}H] formed was separated from the remaining dehydrocyclopeptine-[G-^{3}H] by TLC (Al_2O_3 PF_{254}, MeOH–Me_2CO (1:1) development 4×). The Al_2O_3 at the origin containing the viridicatin-[G-^{3}H] was scraped off and treated with 0.5 ml MeOH, 0.1 ml 2 M KOH and 0.1 ml 30% H_2O_2 at room temp. (cf. [7]). After 12 hr the radioactivity of the mixture was determined. By calculation of DE activity account was taken of the fact that during extraction 30% of the viridicatin-[G-^{3}H] is lost.

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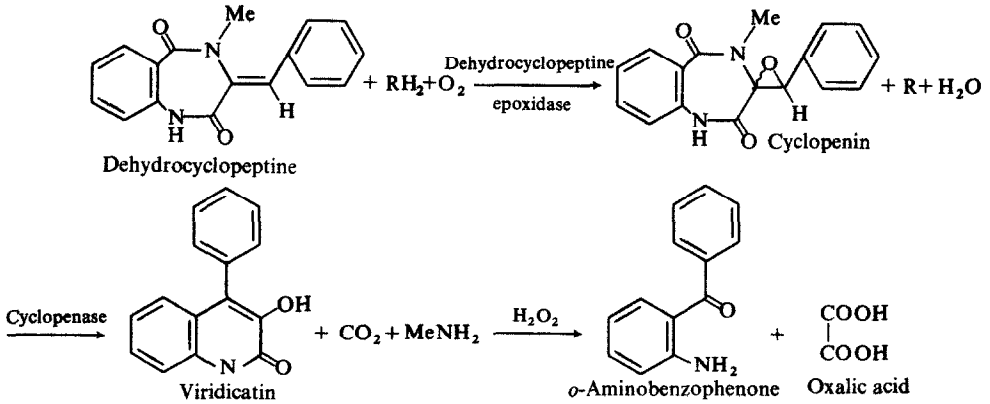

Scheme 1. Epoxidation of dehydrocyclopeptine and transformation of the product cyclopenin into 2-aminobenzophenone.

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