Phytochemistry, 1975, Vol 14, pp 2573–2577 Pergamon Press Printed in England

CYCLOPEPTINE DEHYDROGENASE IN *PENICILLIUM CYCLOPIUM**

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(*Revised received* 15 *June* 1975)

Key Word Index—*Penicillium cyclopium*; Aspergillaceae, Ascomycetes, growth phases, cyclopeptine dehydrogenase, benzodiazepine alkaloids, biosynthesis; NAD(P)-dependent enzyme, flavins

Abstract—Cyclopeptine and dehydrocyclopeptine, intermediates of cyclopenin biosynthesis in *Penicillium cyclopium*, can be reversibly transformed by homogenates of this fungus which contain cyclopeptine dehydrogenase. The enzyme can be assayed spectrophotometrically in the system $NAD(P)^+$/NAD(P)H or by linking to diaphorase/2,6-dichlorophenolindophenol. While X-Press and acetone treatments of the mycelium are the most suitable disruption methods for assaying the enzyme on an analytical scale, grinding with sand proved more suitable for preparative work. Part of the total enzyme activity in the hyphae as well as in the conidiospores, is found in the cell wall-protoplasmic membrane-fraction. The soluble portion of the enzyme was 98-fold enriched. Cyclopeptine dehydrogenase activity increased at the beginning of the alkaloid-production-phase, indicating that the enzyme is concerned in alkaloid metabolism.

INTRODUCTION

Isotope experiments have recently shown that cultures of *Penicillium cyclopium* form the benzodiazepine alkaloids cyclopenin and cyclopenol via cyclopeptine and 3,10-dehydrocyclopeptine. Moreover, the latter intermediates could be isolated from alkaloid-producing cultures and are reversibly transformed into each other *in vivo* [1]. The present paper reports on the enzyme cyclopeptine dehydrogenase which catalyzes this reaction.

RESULTS AND DISCUSSION

The presence of cyclopeptine dehydrogenase in extracts of fungal cells was shown by the formation of dehydrocyclopeptine and NAD(P)H from cyclopeptine and $NAD(P)^+$ and vice versa (Fig. 1). The benzodiazepine derivatives formed during these reactions, after separation by TLC, were found to be identical with the products of alkaloid synthesis *in vivo*. Cyclopeptine dehydrogenase activity may be conveniently assayed by determination of the NAD(P)H formed during the reduction of cyclopeptine, either directly (A at 340 nm) or after coupling with the system 2,6-dichlorophenolindophenol/diaphorase (NADH: acceptor oxidoreductase, E.C. 1.3.99.3). The purified enzyme was found to use NAD^+/NADH as well as $NADP^+$/NADPH. However, the reduction of NAD^+ is 50% faster than that of $NADP^+$. The reaction is optimal in tris–HCl buffer (50 mM, pH 9·1) in the presence of 10 mM Mg^{2+} at 30°.

X-Press homogenates have higher cyclopeptine dehydrogenase activity than those resulting from

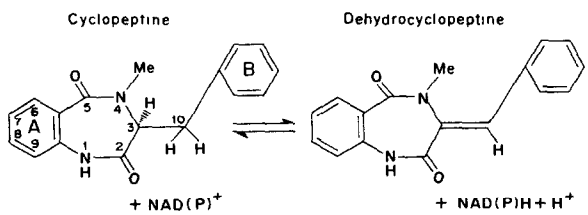


Fig 1 The cyclopeptine dehydrogenase reaction

* Abstracted from a Ph D Thesis submitted by E Aboutabl to Martin-Luther-University, Halle-Wittenberg, DDR

Table 1 Cyclopeptine dehydrogenase activity in cell preparations of *P cyclopium*

Method of cell disruption	Cyclopeptine dehydrogenase activity mU/culture Homogenate	mU/culture Supernatant (1400 *g*)	mU/mg protein Homogenate	mU/mg protein Supernatant (1400 *g*)	% in the cell wall/protoplasmic membrane fraction of the enzyme activity of the homogenates
Grinding with sand (1 hr)	1081*	625	1 87	3 32	42
Grinding with alcoa (1 hr)	902*	352	1 67	1 32	61
Freezing and thawing (5 cycles)	752*	135	1 93	1 18	82
Treatment with acetone	1363	154	2 6	2 22	89
X-Press	1645	517	1 92	3 6	69

* Still containing whole cells

acetone treatment (Table 1). By both of these methods, the hyphae as well as the conidiospores [2] are disrupted and they are therefore suitable for a quantitative assay of cyclopeptine dehydrogenase activity. Grinding with sand, on the other hand, allows mechanical disruption of large amounts of material. For this reason, this method is appropriate for preparative work on the soluble enzyme A lower cyclopeptine dehydrogenase activity was obtained on grinding with other abrasives or on freezing/thawing, due to the fact that these methods fracture only a small proportion of the spores [2]

The benzodiazepine alkaloids cyclopeptine, dehydrocyclopeptine, cyclopenin and cyclopenol are synthesized by the idiophase hyphae as well as by the conidia [3]. Moreover, cyclopeptine dehydrogenase was detected in both these cell types In batch cultures, cyclopeptine dehydrogenase activity increases strongly at the beginning of the alkaloid production phase (idiophase), reaching its maximum with the maximum of cyclopenin/cyclopenol formation (Fig 2). The rates of excretion of cyclopeptine and dehydrocyclopeptine by the hyphae are similar [1]. This, together with the prevention of further increase of alkaloid formation by addition of inhibitors of gene expression (e g cycloheximide) [4], indicates that the enzymes of alkaloid biosynthesis at that time are formed *de novo*

No method of disintegration tested solubilized all cyclopeptine dehydrogenase activity (Table 1). Fractional centrifugation showed that (as with cyclopenase, an enzyme localized on the inner side of the protoplasmic membrane of the conidiospores [5]) a high percentage of cyclopeptine dehydrogenase activity is found in the fraction containing the cell wall together with the tightly-bound protoplasmic membrane In the mitochondrial fraction (containing the bulk of the reference enzyme succinic dehydrogenase; EC.1.3 99 1) as well as in the soluble fraction only small amounts of cyclopeptine dehydrogenase activity were found. However, it is still an open question

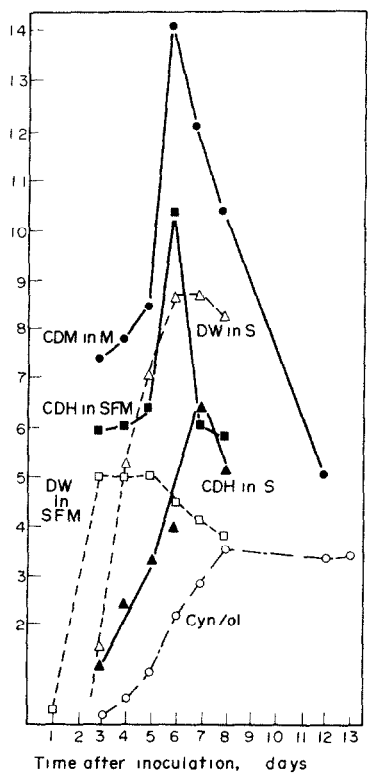


Fig 2 Dynamics of cyclopeptine dehydrogenase activity (CDH), Cyclopenin/cyclopenol-production (Cyn/ol) and dry wt (DW) in whole mycelium (M), spore-free mycelium (SFM) and conidiospores (S) during the growth of cultures of *P cyclopium* 10 = 400 μg Cyn/ol per ml culture filtrate, 6 mg DW and 5 mμ CHD per cm² culture area, respectively

Table 2 Enrichment of the soluble part of cyclopeptine dehydrogenase

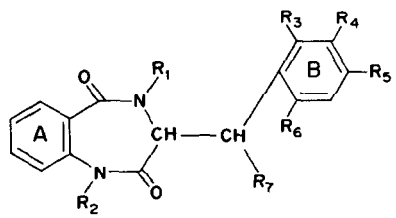
Enrichment step	Protein (mg)	Cyclopeptine dehydrogenase activity Total (mU)	Specific mU/mg protein	Enrichment factor	Yield %
1 Supernatant (22000 *g*) after disintegration with sand	738	1080	1,6	1	100
2 Ammonium sulphate fractionation (30–80% saturation)	10·5	780	74	46	72
3 Ethanol fractionation (30–80%)	5 1	576	115	72	53
4 DEAE Sephadex A_{25} chromatography	2·0	314	157	98	29

whether the enzyme is bound to cell structures *in vivo*.

Nearly 100-fold purification of the enzyme is possible in 3 steps (Table 2). With the purified preparation the usual Michaelis-Menten relationship was demonstrated, giving K_m values for cyclopeptine and NAD^+ of $1\,6 \times 10^{-3}$ M and $2{\cdot}8 \times 10^{-4}$ M respectively. Under V_{max} conditions, the reaction with cyclopeptine is 1·7 times more rapid than with NAD^+.

Cyclopeptine dehydrogenase showed a high degree of substrate specificity, especially regarding position 3 of the cyclopeptine molecule since only the naturally occurring 3-S isomer is attacked. By testing analogues (Table 3), the enzyme was shown to be inactive towards the 10-hydroxy derivative of cyclopeptine (No. 15) indicating the importance of the presence of the $-CH-CH_2-$ group in the molecule. Also other alterations of the cyclopeptine structure reduce the enzymatic transfor-

Table 3 Transformation of S,R-cyclopeptine analogues by cyclopeptine dehydrogenase



Compound	Substituent R_1	R_2	R_3	R_4	R_5	R_6	R_7	Rate of transformation S,R-Cyclopeptine = 100
1*	Me	H	H	H	H	H	H	100
2	H	H	H	H	H	H	H	60
3	CH_2Ph	H	H	H	H	H	H	10
4	H	Me	H	H	H	H	H	60
5	Me	H	H	H	Cl	H	H	40
6	H	H	H	H	Cl	H	H	20
7	Me	H	Cl	H	H	H	H	25
8	Me	H	H	Cl	Cl	H	H	7
9	Me	H	Cl	H	H	Cl	H	0
10	Me	H	H	H	OH	H	H	0
11	Me	H	H	OH	H	H	H	0
12	Me	H	H	H	OMe	H	H	0
13	Me	H	H	OMe	H	H	H	0
14	Me	H	OMe	H	H	H	H	0
15	Me	H	H	H	H	H	OH	0

* Cyclopeptine

Table 4. Effect of activators and inhibitors on cyclopeptine dehydrogenase activity

Effector	Concentration 10^{-3} M	10^{-4} M	Effector	Concentration 10^{-3} M	10^{-4} M
Lead acetate	−60	−28	Zinc sulphate	+4	0
Cadmium sulphate	−58	−19	Borax	+7	0
Calcium chloride	−14	−5	Sodium acetate	+8	0
Cobalt sulphate	−47	−17	Sodium fluoride	−11	−4
Copper sulphate	−57	−23	Trisodium phosphate	+4	0
Magnesium chloride	+26	+20	EDTA	−8	0
Manganese chloride	+5	0	Cysteine	+12	+5
Nickel sulphate	−57	−26	Reduced glutathione	+48	+19
Mercuric chloride	−80	−48	Dithiothreitol	+72	+31
Silver nitrate	−71	−39			

Activation (+) and inhibition (−) in % of the value without effector.

mation partially or totally. Since cyclopeptine *m*-hydroxylated in ring B (No. 11) is not dehydrogenated, the *m*-hydroxy group which is a characteristic of the alkaloids cyclopenol and viridicatol produced by cultures of *P. cyclopium* must be introduced at a later step of the biosynthetic pathway. This agrees with the recent demonstration of an enzymatic activity which transforms cyclopenin into cyclopenol *in vivo* [6] and *in vitro* [7].

Cyclopeptine dehydrogenase purified by $(NH_4)_2SO_4$ fractionation at pH 6 can be reactivated by FAD. Moreover, acid-dissociation of the purified enzyme preparations resulted in the liberation of a flavin which was chromatographically identical with FAD. These data indicate that the enzyme is a flavoprotein. In connection with this, it was found that FAD is the main riboflavin derivative in the mycelium, reaching its maximal content on the fifth day of growth whereas in the culture filtrate of batch cultures, riboflavin (apart from traces of FMN and FAD) continuously accumulates.

The effect of various activators and inhibitors of cyclopeptine dehydrogenase is illustrated in Table 4. The enzyme is inhibited by heavy metals, *p*-chloromercuribenzoate and iodacetamide, while being activated by Mg^{2+}, cysteine, reduced glutathione and dithiothreitol. The inhibition caused by *p*-chloromercuribenzoate and iodacetamide is partially reversed by compounds containing SH-groups viz. cysteine, reduced glutathione and dithiothreitol, suggesting that SH-groups are important for the activity of the enzyme.

EXPERIMENTAL

The strain of *P. cyclopium* Westling (SM 72) originated from a culture of *P. cyclopium* LSHTM No. 72 [8–10]. Culture conditions were as described in ref. [8]. Cyclopeptine in racemic and optically active (3S- and 3R-) forms as well as its structural analogues were synthesized by Dr. Aida Elazzouny [1,11]. Separation of hyphae and conidiospores, estimation of dry wt, estimation of protein and cyclopenin/cyclopenol were performed as described in ref. [1], chromatographic separation of cyclopeptine and dehydrocyclopeptine as described in ref. [1]; extraction, purification, identification and estimation of flavins as given in ref. [12]; estimation of cyclopenase and succinic dehydrogenase as in refs. [2] and [13], respectively.

Cell disruption. Cells of P. cycloplium were suspended in 2 vol tris–HCl buffer (50 mM, pH 7·5) using a knife-homogenizer. The suspension was then mixed with 2 parts of sand and ground (1 hr) at 0° in a mortar. The mechanical disruption under pressure was performed using the Biotec X-Press equipment (Bromma, Sweden). The cell suspension at −30° was pressed 5× and allowed to thaw at 4°. Me_2CO dry powder was prepared by dropwise-addition of 1 vol of cell-suspension to 9 vols Me_2CO at −20° with continuous stirring. After 5 min, the mixture was filtered and the residue washed with cold Me_2CO and allowed to dry at 4°. Freezing and thawing was carried out by freezing the cell-suspension in MeOH/dry ice (−70°) and slowly thawing at 9°. The process was repeated 5×.

Cell fractionation. The disrupted cell material was subjected to differential centrifugation at 1400 *g* for 30 min (cell wall–protoplasmic membrane fraction) and 22000 *g* for 30 min (mitochondrial fraction) respectively. At each step, the sediment was washed with a small amount of the medium and recentrifuged. Honda's medium was used for cell disintegration and for the fractionation procedure [15].

Purification of cyclopeptine dehydrogenase. A cell-free extract prepared by grinding 6-day-old fresh mycelium with sand and tris–HCl buffer (50 mM, pH 7·4) at 0° and successive centrifugation at 4000 *g* and 22000 *g*, was treated with $(NH_4)_2SO_4$ (0·3–0·8 saturation) and the pH adjusted to 7. The ppt. was dissolved in 50 ml tris–HCl buffer and subjected to fractional precipitation (30–80%) with EtOH (−20°). The ppt. was freed from solvent, dissolved in 5 mM tris-HCl buffer (pH 7·2) and subjected to chromatography on a DEAE-Sephadex A_{25} column. Elution was started using a linear gradient of 0–1 M NaCl in the above tris buffer.

Estimation of cyclopeptine dehydrogenase activity. For measurement of the NAD(P)H at 340 nm, the incubation-mixture contained 5 μmol cyclopeptine, 8 μmol NAD^+, enzyme soln, tris–HCl buffer (50 mM, pH 9·1) and H_2O to 2 ml. If the NAD(P)H was indirectly determined, diaphorase (*ca* 1 U,

prepared according to ref [14]) and 2,6-dichlorophenolindophenol (0·2 μmol) were additionally included and tris–HCl buffer (50 mM, pH 8) was used. For assay of crude homogenates by the UV-method, KCN (1·5 μmol) was added to inhibit mitochondrial enzymes and incubates were shaken at 30° for 30 min. The reaction was started by the addition of the enzyme and terminated by the addition of 2 ml MeOH. 1 unit of cyclopeptine dehydrogenase was calculated as that amount of enzyme which catalyzes the dehydrogenation of one μmol of cyclopeptine/min under the conditions described.

Acknowledgements—E. Aboutabl thanks the Ministries of High Education in the ARE and GDR for organizing a leave of absence from the Faculty of Pharmacy, Cairo University, and a research programme at the Section of Pharmacy, Martin-Luther-University, Halle-Wittenberg.

REFERENCES

1. Framm, J., Nover, L., El Azzouny, A., Richter, H., Winter, K., Werner, S. and Luckner, M. (1973) *European J. Biochem* **37**, 78.
2. Wilson, S., Schmidt, I., Roos, W., Furst, W. and Luckner, M. (1974) *Z. Allgem. Mikrobiol.* **14**, 515.
3. Nover, L. and Luckner, M. (1974) *Biochem. Physiol. Pflanzen* **166**, 293.
4. El Kousy, S., Pfeifer, E.,Ininger, G., Roos, W., Nover, L. and Luckner, M. (1975) *Biochem. Physiol. Pflanzen* **168**, 79.
5. Wilson, S. and Luckner, M. (1975) *Z. Allgem. Mikrobiol.* **15**, 45.
6. Nover, L. and Luckner, M. (1971) *Abh. Deut. Akad. Wiss. Berlin* **1971** 535.
7. Luft, I. (1974) *Pharmazie* **29**, 73.
8. Nover, L. and Luckner, M. (1969) *European J. Biochem.* **10**, 268.
9. Bracken, A., Pocker, A. and Raistrick, H. (1954) *Biochem. J.* **57**, 587.
10. Birkinshaw, J. H., Luckner, M., Mohamed, Y. S., Mothes, K. and Stickings, E. C. (1963) *Biochem. J.* **89**, 196.
11. Elazzouny, A. (1974) Ph.D. thesis, Martin-Luther-University, Halle/S., DDR.
12. Fazekas, A. G. and Kokai, K. (1971) in *Methods in Enzymology*, Vol. 18, Part B, McCormick, D. B. and Wright, L. D. (Eds.) p. 385, Academic Press, New York.
13. Slater, E. C. (1964) in Hoppe-Seyler/Thierfelder "*Handbuch der physiologisch- und pathologisch-chemischen Analyse*, 10th Ed. Vol. 6, part 1, 636, Springer-Verlag, Berlin.
14. Davies, D. D., Teixeria, A. and Kenworthy, P. (1972) *Biochem. J.* **127**, 335.
15. Honda, S. I., Hongladarom, T. and Wildman, S. G. (1962) *Plant Physiol.* **37**, XLI.