THE ENZYMIC FORMATION OF PENICILLIC ACID

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Received 10 March 1975

1. Introduction

The metabolic reaction sequence for the formation of penicillic acid from orsellinic acid in *Penicillium cyclopium* has recently been elaborated in our laboratory [1]. Labelling experiments indicated that the last step in this sequence is an oxidative ring fission of 3-methoxytoluquinone. In the present communication we describe the isolation of an enzyme complex that can catalyze the ring cleavage and the formation of penicillic acid. Besides 0_2 the enzyme complex requires NADPH. It is stimulated by FMN and Fe^{2+}.

2. Methods

Penicillium cyclopium, NRRL 1888 was grown for 72 hr in 500 ml conical flasks on a shake table at 28°C. Each flask contained 175 ml of Raulin–Thom medium supplemented with diammonium tartrate as described by Bently and Keil [2]. The filtered mycelium was thoroughly washed with 0.1 M Tris–HCl buffer pH 8.0. Washed, frozen mycelium could be stored up to 3 weeks without significant loss of enzymic activity. The cells were disrupted by treatment with glass beads in a mechanical homogenizer for 20 min below 10°C. The homogenate was filtered through gauze and the remaining cell debris removed by centrifugation at 3 000 *g* for 20 min at 4°C. The supernatant was recentrifuged at 105 000 *g* for 40 min. After passing the clear solution through Sephadex G-25 equilibrated with the Tris–HCl buffer this preparation was used in the described experiments. The enzyme activity was measured spectrophotometrically by the rate of decrease in absorbance at 340 nm in a cuvette with 1-cm light path containing in 2.0 ml Tris–HCl, pH 8.0, 10^{-1} M; NADPH $20–100 \cdot 10^{-6}$ M; FMN $20 \cdot 10^{-6}$ M; Fe^{2+} 10^{-3} M; methoxytoluquinone $10 \cdot 10^{-6}$ M; enzyme solution 0.50 ml.

When penicillic acid formation was measured the incubation was performed with 5.0 ml of the described reaction mixture and [^{14}C]H_3O–toluquinone [1].

Penicillic acid was isolated by extraction of the acidified incubation solution with 5 ml ethylacetate. The unreacted quinone was measured spectrophotometrically at 258 nm and penicillic acid subsequently extracted with aqueous $NaHCO_3$. After acidification and reextraction with ethylacetate the substances were separated by thin-layer chromatography (the organic phase of the silica gel; mixture chloroform–methanol–water–formic acid, 250:24:25:1, by vol). Penicillic acid was scraped off the plate and its radioactivity was determined in a scintillation spectrometer after extraction from the silica gel.

In the stoichiometric measurements the oxygen consumption was determined by the use of a Clark electrode.

3. Results and discussion

The overall reaction from methoxytoluquinone to penicillic acid requires 0_2 and NADPH and is stimulated on the addition of FMN and Fe^{2+} (table 1). NADH is less effective as an electron donor than NADPH. The pH dependence of the reaction has been tested over the range from 5 to 9 and optimal enzyme activity was obtained at pH 8.0. The centrifugation at 105 000 *g* and the subsequent treatment with Sephadex G-25 gives 5 times enrichment of the enzyme complex.

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Table 1
Enzymic penicillic acid formation (complete incubation mixture 5 ml, Tris–HCl, pH 8.0 10^{-1} M, NADPH $30 \cdot 10^{-6}$ M, FMN $20 \cdot 10^{-6}$ M, Fe^{2+} 10^{-3} M, [^{14}C]H_3O–toluquinone $10 \cdot 10^{-6}$ M, enzyme solution 1.0 ml)

Incubation mixture	Penicillic acid (dpm)
Complete	28 800
– Fe^{2+}	21 000
– Fe^{2+}–FMN	20 100
+ EDTA ($2 \cdot 10^{-3}$ M)	25 200
+ bipyridyl ($2 \cdot 10^{-3}$ M)	7200

The enzyme complex can be brought to further purification by ammonium sulfate fractionation and separation on Sephadex G-75 or G-150. During these purification steps, however, the enzymes participating in the overall reaction separate and have to be studied individually. At present only studies on the nonfractionated enzyme mixture is reported.

When the enzyme incubation is performed anaerobically no NADPH is consumed indicating that the quinone is not reduced to the hydroquinone state prior to the ring opening reaction. Furthermore, no NADPH is oxidized in absence of the quinone derivative. The hydroquinone is slowly utilized as a substrate depending on air oxidation to the quinone. The overall reaction is not inhibited by EDTA but 2,2′-bipyridyl (2 mM) causes 75% inhibition which confirms the essential role of Fe^{2+} in the reaction.

The stoichiometry of the reaction measured spectrophotometrically on NADPH and quinone consumption respectively indicates the utilization of 2 moles of NADPH per mole of the quinone. The oxygen measurement showed that oxygen and quinone are used in equimolar amounts (table 2). The overall reaction can thus be described with the following equation:

$$2\ \text{NADPH} + 2\ H^+ + O_2 + \text{quinone} \rightarrow 2\ \text{NADP}^+ + H_2O + \text{penicillic acid}$$

The mechanism for the total reaction can be formulated in more than one way. The most straightforward alternative is outlined in fig.1 in which a

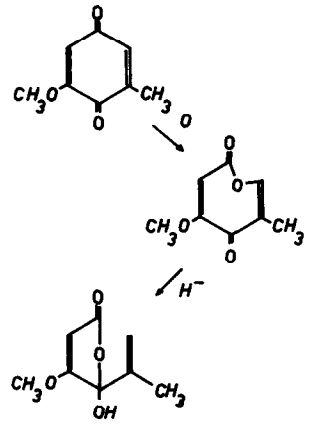

Fig.1. Suggested mechanism for the terminal reactions in penicillic acid formation.

Baeyer–Williger type of oxidation of the quinoid ring is suggested. Rearrangement of the intermediate lactone and a concerted hydrogenation of the formylic carbon gives immediately penicillic acid.

In the suggested reaction mechanism the oxygen is activated through the NADPH–FMN–Fe^{2+} system. This mechanism is analogous to the monooxygenase action described by Conrad et al. [3] for the oxidation of D(+)-camphor to 1,2-campholide. These authors are able to separate the monooxygenase complex in a NADH dehydrogenase and a lactonase. The similarity between the penicillic acid forming system and the camphor lactonizing system is further emphazised by preliminary experiments which indicate

Table 2
Stoichiometry of NADPH, oxygen and methoxytoluquinone in penicillic acid formation

Exp.	NADPH / quinone	quinone / oxygen
	Mole ratio	
1	1.80	0.95
2	2.25	1.20
3	2.90	
4	2.05	
5	1.80	
Mean value	2.16	1.07

that the corresponding separation is achievable also for the quinone oxidizing complex.

The suggested mechanism for cleavage of aromatic rings via a quinoid structure is theoretically applicable to the formation of a number of natural products. Examples of such substances are patulin, sulochrin, sterigmatocystin, and aflatoxins.

Acknowledgement

This work was supported by grants from the Swedish Natural Science Research Council

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