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SHORT COMMUNICATION

FACTORS AFFECTING THE INDUCTION OF 11α-HYDROXYLASE OF PROGESTERONE IN THE FILAMENTOUS FUNGUS *RHIZOPUS NIGRICANS*

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Summary—The 11α-hydroxylase of progesterone was induced in the filamentous fungus *Rhizopus nigricans* ATCC 6227b with different steroids as inducers and the induction process was optimized in regard to the age of the mycelium, to the concentration of the inducer and to the time of induction. Deoxycorticosterone and testosterone, steroids with higher polarity of the side-chain than progesterone, although poorer substrates for *in vivo* hydroxylation than progesterone, induced more enzyme compared to progesterone. Other alterations in the steroidal ring system examined diminished the induction capability of the inducing steroid to different extent.

The highest 11α-hydroxylating activity, if expressed on the basis of mycelial wet weight, was achieved with 18 h old mycelium which was induced for 2 h with 0.30 mM deoxycorticosterone.

INTRODUCTION

The 11α-hydroxylase of progesterone is in the filamentous fungus *Rhizopus nigricans* ATCC 6227b (*R. nigricans*) induced by progesterone [1–3], the induction being greatly dependent on the degree of saturation with oxygen [3]. It was shown that the hydroxylase represented an electron-transport chain carrying electrons from NADPH to cytochrome *P*-450 which received electrons from an iron-sulfur protein (rhizoporedoxin oxido-reductase) [4–6].

In our studies of the 11α-hydroxylase from *R. nigricans* we are interested in the mechanism of induction, on the one hand, and in the mechanism of the hydroxylation reaction studied by reconstitution of purified constituents, on the other hand. For these purposes the enzyme constituents are needed in purified form and in sufficient amounts. It was, therefore, important to determine optimal conditions for the induction of the 11α-hydroxylation system, since all three constituents of the electron-transport chain seemed to be inducible [1, 7].

The studies of bioconversions of different mono-oxo- and di-oxo-4 steroids with *R. nigricans* indicated [8] in view of the discovery that the 11α-hydroxylase was an inducible enzyme system, that progesterone was not the only inducing steroid in this fungus. Quantitative data on how good an inducer a steroid was, were however, missing.

The nature of several factors affecting the extent of the hydroxylase induction was suggested by studies of other steroid transforming microorganisms [8–10]. These factors included the concentration of the inducer, the time of induction and the age of mycelium.

In the present study, we investigated the induction of the 11α-hydroxylase with a series of structurally and functionally different steroids and we optimized the conditions of induction in regard to the factors cited above.

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EXPERIMENTAL

Materials

Rhizopus nigricans ATCC 6227b was cultivated as described previously [4].

The chemicals were obtained from the following companies: [1,2,6,7^{3}H]progesterone (101 Ci/mmol)—Amersham International (Amersham, England), cycloheximide, glutathione and steroids—Sigma Chemical Company (St Louis, Mo.); Bacto agar and casamino acids—DIFCO (U.S.A.). All other chemicals were analytical grade from BDH Chemicals Ltd. (Dorset, England).

Enzyme induction

R. nigricans was grown in nutrient medium [4] for intervals of time indicated in the text, washed with cold distilled water and dried between filter paper; 4 g portions of moist mycelia were resuspended in 100 ml prewarmed (28°C) phosphate buffer (1 mM sodium phosphate, 0.2 mM EDTA, 0.04 mM reduced glutathione, pH 5.5) supplemented with 0.2% (w/v) casamino acids. The steroids were dissolved in dimethyl formamide and added to the resuspended mycelium at 0.30 mM final concentration. The induction proceeded for 3 h if not otherwise stated.

Assay for the hydroxylation activity

2 g moist mycelia were washed with saline [7] and resuspended in 30 ml phosphate buffer in the presence of cycloheximide and progesterone as substrate [1, 2] and vigorously shaken at 28°C for different intervals of time depending on the induced enzyme activity. The reaction products were extracted with chloroform and analyzed as described [6]. [^{3}H]progesterone was used as substrate in those assays in which hydroxylation activity was so low that the products

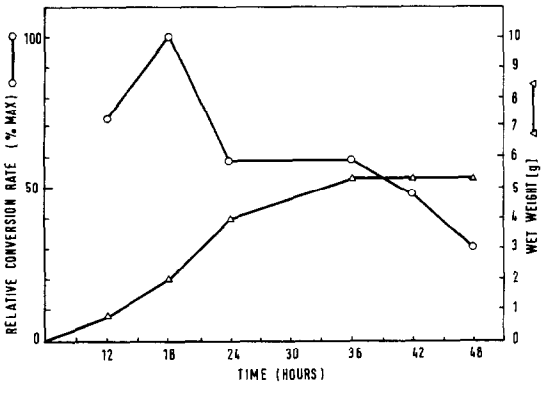

Fig. 1. The effect of age of the fungal mycelium on its ability to transform progesterone to 11α-hydroxyprogesterone. The fungal mycelium was grown for different intervals of time, induced for 2 h with progesterone in buffer supplemented with casamino acids and assayed for hydroxylation activity as described in Experimental.

were not detectable with the standard method listed above [6]. In these instances 0.5 g moist mycelia were incubated under standard conditions with 30 nmol/ml radioactively labelled progesterone. After TLC performed on precoated Silica-Gel plates (Merck, Darmstadt, Germany) the labelled metabolites were located with Packard Radiochromatogram scanner and per cent conversion calculated from the areas under the corresponding radioactivity peaks. The conversion of progesterone was followed after different intervals of time and the enzyme activity was estimated from the tangent to the conversion curve.

RESULTS AND DISCUSSION

Maximal hydroxylation rate after induction with progesterone was 23–46 mg 11α-progesterone/g (dry wt)/h; it varied between different inocula and therefore, only induction with steroids from one inoculum could be compared. Similar observations were made by Wacker *et al.*[10] who studied the induction of 20β-hydroxysteroid:NAD-oxidoreductase by steroids in *Streptomyces hydrogenans*.

The constitutive level of the hydroxylase (Table 1) was 500–1000 times lower compared to the level achieved after induction of the enzyme system with progesterone. When the hydroxylation rate after induction with different steroids was compared it was shown that deoxycorticosterone and testosterone were better inducers than progesterone, although they were shown to be *in vivo* poorer substrates for 11α-hydroxylase than progesterone [11]. Introduction of an oxygen function at C-11 markedly diminished the extent of enzyme induction and aromatization of ring A nearly abolished the inducing capability of the steroid. The induction process does not seem to be very specific (Table 1); all tested steroids induced 11α-hydroxylase to some extent.

Table 1. Relative hydroxylation rates of progesterone after induction of *Rhizopus nigricans* with different steroids as inducers

Inducer	Relative hydroxylation rate
None	<0.2
Progesterone	100
Deoxycorticosterone	200
Testosterone	130
17α-Hydroxyprogesterone	30
9α-Fluoro-16-methyl-prednisolone	35
19-Nortestosterone	22
11-Oxo-progesterone	15
11α-Hydroxyprogesterone	7
Oestrone	2

The fungal mycelium was induced with the corresponding steroid (100 μg/ml final conc.) at 28°C for 3 h by shaking in phosphate buffer supplemented with 0.2% casamino acids. 2 g Moist mycelia were assayed for the hydroxylation activity with 100 μg/ml progesterone as substrate in the presence of cycloheximide (100 μg/ml). The hydroxylation rate was determined from the tangent of the conversion curve as stated in Experimental.

As shown in Fig. 1 the highest yield of the enzyme was achieved when mycelium was induced 18 h after inoculation of spores what corresponds in *R. nigricans* to the mid-logarithmic phase of growth. The induction was complete after 2 h aeration in the presence of inducer (Fig. 2A) in agreement with our data where progesterone was used as inducer [2]. Optimal induction was achieved at 100 μg/ml (3×10^{-4} M) steroid as shown in Fig. 2B. At higher concentrations of the inducing steroid less hydroxylase was induced regardless of the steroid used as inducer (data not shown). This observation could be explained by interference of the steroids in the medium with the membrane transport system of the cell.

A comparison of *R. nigricans* with other steroids transforming microorganisms reveals that the optimal age of mycelium differs even between closely related fungi; comparing *R. nigricans* and *R. arrhizus* the optimal ages of mycelium for induction were 18 h and 36 h, respectively [13]. The time of induction was similar in *R. nigricans*, *R. arrhizus* [13] and *Curvularia lunata* [12], but was quite different from *Fusarium solani* [14] and *Aspergillus*

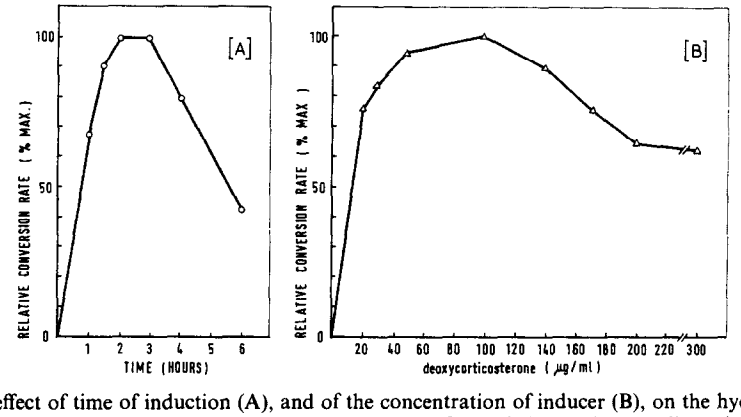

Fig. 2. The effect of time of induction (A), and of the concentration of inducer (B), on the hydroxylation activity in *Rhizopus nigricans*. (A) *R. nigricans* was grown for 42 h in nutrient medium, induced with deoxycorticosterone for different periods of time and hydroxylation rate determined as described in Experimental. (B) Fungal mycelia were grown as described in Fig. 2A and induced with deoxycorticosterone at different concentrations. Hydroxylation rate was estimated as described in Experimental.

ochraceus [8], where the time of induction was 18 h and 14 h, respectively.

Structural differences between steroids which favored the induction could be attributed to a more polar side-chain. It seems, however, that this is not the whole answer, since the introduction of the α-hydroxyl group at C-17 of progesterone resulted in a lower enzyme level presumably due to steric hindrance at C-17. It was surprising that the absence of the angular methyl group at C-19 (19-nortestosterone) reduced enzyme induction, suggesting that a hydrophobic group on that side of the steroid molecule is important for the induction mechanism. The fact that the induction process of the 11α-hydroxylase is in *R. nigricans* relatively nonspecific in respect to the inducing steroid is in agreement with an induction mechanism which involves protein receptors exhibiting different affinities towards different inducing steroids. Investigations with the aim to prove such a hypothesis are in progress.

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