6 β -Hydroxylation of Steroids by Extracts of *Aspergillus niger*

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Growing cultures of *Aspergillus niger* hydroxylate progesterone at the 6 β and 11 α positions. The properties of the 6 β -hydroxylase activity were studied in extracts of this organism using 11 α -hydroxyprogesterone as substrate. Maximum 6 β -hydroxylase activity was obtained at pH 6.5. EDTA and thiophenol inhibited hydroxylation, while NADPH, 2-oxoglutarate and pyruvate stimulated it. Cobalt and cadmium ions inhibited the 6 β -hydroxylase activity. The feasibility of introducing a hydroxyl group into the 6 β -position of various steroid compounds was tested: the 3-oxo- Δ^4 configuration appeared to exert a directional effect for 6 β -hydroxylation, while an 11 β -hydroxyl group exerted some kind of steric hindrance against 6 β -hydroxylation.

INTRODUCTION

The important discovery by Peterson & Murray (1952) of the microbiological 11 α -hydroxylation of progesterone and other steroids led to the use of a variety of micro-organisms for hydroxylating every position in the steroid nucleus. However, steroid hydroxylation by extracts of micro-organisms has been described in only a few instances. Zuidweg *et al.* (1962) prepared an extract from *Curvulária lunata* capable of catalysing the hydroxylation of Reichstein's compound S to hydrocortisone. A 9 α -hydroxylase activity was demonstrated in extracts of *Nocardia restrictus* by Chang & Sih (1964). Wilson & Vestling (1965) obtained an extract from *Bacillus megaterium* KM able to hydroxylate deoxycorticosterone mainly at the 15 β position. Zuidweg (1968), using extracts of *C. lunata*, studied the nature of the hydroxylation of Reichstein's compound S at the 11 β and 14 α positions. Sallam *et al.* (1971), using a cell homogenate of *Rhizopus nigricans* REF 129, studied the hydroxylation of progesterone at the 11 α , 17 α and 21 positions. Nguyen-Dang-Tam *et al.* (1971) showed that extracts of *Aspergillus niger* catalysed the hydroxylation of progesterone at the 11 α position.

Previously, I have studied the hydroxylation of progesterone at the 11 α and 17 α positions by extracts of *Rhizopus nigricans* NRRL 1477 (El-Kady & Allam, 1973; Allam & El-Kady, 1975). In this report, I describe an investigation of the 6 β -hydroxylation of progesterone and 11 α -hydroxyprogesterone by extracts of *Aspergillus niger*.

METHODS

Cultures. *Aspergillus niger* isolate no. 58 (from the Culture Collection of the Mycological Laboratory, Botany Department, Assiut University, Egypt) was cultivated in 250 ml Erlenmeyer flasks which contained 50 ml of the medium described by Capek *et al.* (1964). The pH was adjusted to 5.6. After sterilization, each flask was inoculated with 2 ml of a spore suspension made from a 1-week old culture. The inoculated flasks were agitated on a reciprocating shaker at 28 °C. After 24 h growth, the cultures were activated by the addition of 5 mg progesterone per flask and reagitated for another 24 h.

Preparation of extracts. Mycelium was harvested by filtration, washed, thoroughly with 0.5% (w/v) NaCl solution, followed by distilled water, and blotted dry with absorbent paper. This mycelium (15 g) was then ground with cold sand and extracted with 100 ml cold water or 0.1 M-potassium phosphate buffer, pH 6.5. The slurry obtained was centrifuged at 4000 rev. min⁻¹ for 15 min and the supernatant was used as the enzyme source.

Assay of 6 β -hydroxylating activity in extracts. To 5 ml portions of the extracts in 25 ml Erlenmeyer flasks were added 5 mg 11 α -hydroxyprogesterone in 0.1 ml ethanol. The contents were mixed immediately and the flasks were agitated on a reciprocating shaker for 5 h at 30 °C. At the end of this period the contents of each flask were

extracted with 10 ml chloroform. Extraction was repeated three times and the combined chloroform extracts were then treated with 0.5 vol. of 5% (w/v) sodium bicarbonate solution followed by an equal volume of distilled water. The residue was dissolved in a measured volume of chloroform/methanol (1:1, by vol.).

Determination of reaction products. The hydroxylated product in the prepared residue was first identified by conventional thin-layer chromatography with an authentic sample, using cyclohexane/acetone/chloroform (75:25:20, by vol.) as solvent. The amounts of the product and residual substrate were determined by preparative thin-layer chromatography with the same solvent. The bands containing the compounds were scraped off and eluted with ethanol. The concentration of each was then determined by u.v. spectrophotometric measurement.

RESULTS AND DISCUSSION

Out of 40 different isolates from five genera (*Rhizopus*, *Mucor*, *Cunninghamella*, *Cladosporium* and *Aspergillus*) tested for 6 β -hydroxylation activity, *Aspergillus niger* isolate no. 58 was the most active and was chosen for further study. Growing cultures of this organism converted 80% of added progesterone (50 mg per 50 ml medium) to 11 α -hydroxyprogesterone (10 mg) and 6 β ,11 α -dihydroxyprogesterone (30 mg) within 48 h.

Using 11 α -hydroxyprogesterone as a substrate, the stability of the 6 β -hydroxylating activity was tested in extracts stored at 5 and -10°C . Extracts kept at 5°C lost 90% of their activity in 24 h and those kept at -10°C lost 60% of their activity in the same period. After 48 h at either temperature all activity was lost. In earlier studies (Chang & Sih, 1964; El-Kady & Allam, 1973) microbial steroid-hydroxylating systems were shown to be unstable.

Effect of pH. The effect of different pH values on the 6 β -hydroxylating activity was studied. Portions (4 ml) of the extract in distilled water were adjusted to pH values ranging from 5.5 to 9 using phosphate or Tris buffers (500 μmol). Hydroxylation at the 6 β position increased with increase in pH, reaching a maximum at pH 6.5. At this pH, 42% of the added 11 α -hydroxyprogesterone was converted to 6 β ,11 α -dihydroxyprogesterone. At pH values between 7 and 9, the 6 β -hydroxylase activity decreased linearly with pH.

Effect of EDTA. EDTA has been used in most studies of the hydroxylation of steroids by extracts of micro-organisms (Zuidweg *et al.*, 1962; Chang & Sih, 1964; Zuidweg, 1968; Sallam *et al.*, 1971; El-Kady & Allam, 1973). The effect of different concentrations of this compound on the 6 β -hydroxylating activity was tested. The presence of EDTA at a concentration as low as 5 mM inhibited about 30% of the 6 β -hydroxylase activity. Higher concentrations of EDTA were more inhibitory (data not presented). This suggests that a metal cation is involved in the hydroxylation.

Effect of some external electron carriers and thiol compounds. A marked stimulation of 6 β -hydroxylase activity was observed in the presence of NADPH (Table 1). 2-Oxoglutarate and pyruvate had a similar effect, but ascorbate, fumarate and succinate were completely inactive. These results are consistent with earlier reports that 2-oxoglutarate stimulates different hydroxylation reactions (Hayaishi, 1969), and that pyruvate can replace 2-oxoglutarate in some enzymic hydroxylation reactions (Hayaishi, 1969).

The effect of some thiol compounds was also studied (Table 2). Glutathione, at a concentration of 5 mM, stimulated 6 β -hydroxylase activity, while 2-mercaptoethanol, sodium thioglycolate and cysteine.HCl had no effect. On the other hand, thiophenol, at the same concentration, caused some inhibition.

Effect of some metal ions. The 6 β -hydroxylase activity was sensitive to Co^{2+} and Cd^{2+} which inhibited 80% and 60% of the 6 β -hydroxylase activity, respectively (Table 3). Previous reports (El-Kady & Allam, 1973) showed that Co^{2+} completely inhibited both 11 α - and 17 α -hydroxylase activities.

Substrate specificity. The feasibility of introducing a hydroxyl group into the 6 β position of some steroid compounds was tested. The following compounds were equally active as substrates for the 6 β -hydroxylase: progesterone, 11 α -hydroxyprogesterone, 17 α -hydroxyprogesterone, 21-hydroxyprogesterone, 11 α ,17 α -dihydroxyprogesterone, epicortisol, testosterone, 11 α -hydroxy-testosterone and androst-4-ene-3,17-dione. In contrast, the following compounds were found to

Table 1. *Effect of some electron carriers on 6 β -hydroxylation*

Reaction mixtures (total volume 6 ml) contained: extract in 0.1 M-phosphate buffer, pH 6.5, 5 ml; 11 α -hydroxyprogesterone, 5 mg; test compound, 3 mM. They were incubated at 30 °C for 5 h with shaking.

Test compound	6 β ,11 α -Dihydroxyprogesterone formed (mg)
None	2.10
NADPH	3.65
Pyruvate	3.55
2-Oxoglutarate	3.60
Fumarate	2.00
Succinate	2.20
Ascorbate	2.00

Table 2. *Effect of some thiol compounds on 6 β -hydroxylation*

Reaction mixtures and assay conditions were as described in the legend to Table 1, except that the test compounds were added at 5 mM.

Test compound	6 β ,11 α -Dihydroxyprogesterone formed (mg)
None	2.1
Glutathione	3.1
2-Mercaptoethanol	2.0
Sodium thioglycolate	2.2
Thiophenol	1.4
Cysteine .HCl	1.9

Table 3. *Effect of some metal ions on 6 β -hydroxylation*

Reaction mixtures and assay conditions were as described in the legend to Table 1.

Test compound	6 β ,11 α -Dihydroxyprogesterone formed (mg)
None	2.2
ZnSO ₄	2.2
MnSO ₄	2.1
CdSO ₄	0.88
FeSO ₄	2.1
FeCl ₃	2.0
CuCl ₂	2.2
CoCl ₂	0.44
MgCl ₂	2.0

be inactive for the 6 β -hydroxylase: 11 β -hydroxyprogesterone, 11 α -hydroxy-5 α -pregnane-3,20-dione, 17 α -hydroxy-5 α -pregnane-3,20-dione, 5 α -pregnane-3 β ,17 α ,21-triol-20-one, 11 β ,21-dihydroxyprogesterone, cortisol, cholesterol and ergosterol. It was noted that all the active substrates contained the 3-oxo- Δ^4 configuration. Hence, this configuration probably exerts a directional effect for 6 β -hydroxylation. The inactive compounds lack at least one of these two functional groups (3-oxo- Δ^4 -), except 11 β -hydroxyprogesterone, 11 β ,21-dihydroxyprogesterone and cortisol which contain the 3-oxo- Δ^4 configuration but also possess an 11 β -hydroxyl group. Thus, it appears that an 11 β -hydroxyl group exerts some kind of steric hindrance against 6 β -hydroxylation.

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