A stereospecific enzyme of the putative biosynthetic pathway of cardenolides

Characterization of a progesterone 5β-reductase from leaves of *Digitalis purpurea* L

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Received 20 June 1990; revised version received 10 August 1990

Leaves of *Digitalis purpurea* contain an enzyme activity which catalyzes the conversion of progesterone to 5β-pregnane-3,20-dione. Since cardenolides without exception possess a 5β-configuration, 5β-pregnane-3,20-dione can serve as a precursor for this class of secondary metabolites. It is assumed that the enzyme is part of the putative biosynthetic pathway of cardenolides. This enzyme activity was spotted in the soluble fraction of a crude homogenate. Product formation was detected by gas chromatography and by gas chromatography/mass spectroscopy (g.c./m.s.). The enzyme had a pH optimum at 8.0 and an apparent K_m value of 6 μM for progesterone. It required NADPH as a co-substrate with an apparent K_m value of 22 μM. The optimum temperature in vitro was 30°C. The activity was not dependent on monovalent and bivalent cations.

Digitalis purpurea; Progesterone; Progesterone 5β-reductase; Cardenolide

1. INTRODUCTION

In addition to tracer and biotransformation experiments, the enzymic background has to be elucidated, in order to establish a biosynthetic pathway. The spatial distribution of cardiac glycosides in *Digitalis* species was examined in correlation to plant age. Radioimmunoassays of plants showed that the highest content was found in the mesophyll and testa, the lowest in the pith [1].

Tracer experiments with ^{14}C-labelled progesterone using leaves of *Digitalis lanata* revealed that the label was incorporated into cardenolides like digitoxigenin, digoxigenin and gitoxigenin as well as into various 5α- and 5β-configurated pregnane derivatives [2]. Tschesche et al. [3] pointed out that cardenolides appeared after the application of 5β-pregnane-3,20-dione and 5β-pregnan-3β-ol-20-one to leaf pieces of *Digitalis lanata*, whereas no cardenolides were formed from the corresponding 5α-derivatives.

Extracts from rat liver are capable of catalyzing the reduction of many Δ4-3-ketosteroides. The 5α-reductase was located in the microsomal fraction, while the 5β-activity was entirely soluble [4]. In a previous paper we described a progesterone 5α-reductase from cell-suspension cultures of *Digitalis lanata* which was also of microsomal origin [5].

The present report characterizes a soluble enzyme which catalyzes the reaction of progesterone to 5β-pregnane-3,20-dione (Fig. 1). Since all cardenolides are 5β-configurated, this is the first stereospecific enzyme in the biosynthetic pathway of cardenolides. In addition, the next step of the putative pathway, which results in the in vitro formation of 5β-pregnan-3β-ol-20-one, could also be detected in crude enzyme preparations (Fig. 1).

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Bis-Tris; bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane

2. MATERIALS AND METHODS

2.1. *Plant material*

Seeds of *Digitalis purpurea* were obtained from the Botanical Garden of the University of Tübingen. The plants were grown in the greenhouse at 24°C and with a 15 h photoperiod.

2.2. *Enzyme preparation*

Leaves were harvested from 60-day-old plants. They were kept on ice for 10 min and then chopped into small pieces. This material was homogenized in 0.5 g PVP (water-insoluble polyvinylpyrrolidone) and 6 ml buffer (0.1 M Hepes, 2 mM EDTA, 0.25 M sucrose, pH 8.0) per 1 g fresh weight in an Ultra Turrax (Janke and Kunkel, Staufen i. Br., FRG) for 3 × 30 s. The supernatants from 45 000 × *g* (15 min) and 100 000 × *g* (1 h) centrifugations were used for the enzyme assay. Protein concentrations were determined according to Bradford [6]. In order to test the effect of pH, the enzyme preparation was carried out with Bis-Tris (pH 7) and Hepes (pH 7). The pH-values in the assay mixture were adjusted to 5.5–7.0 with Bis-Tris/HCl and 7.0–8.5 with Hepes/KOH.

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00145793/90/$3.50

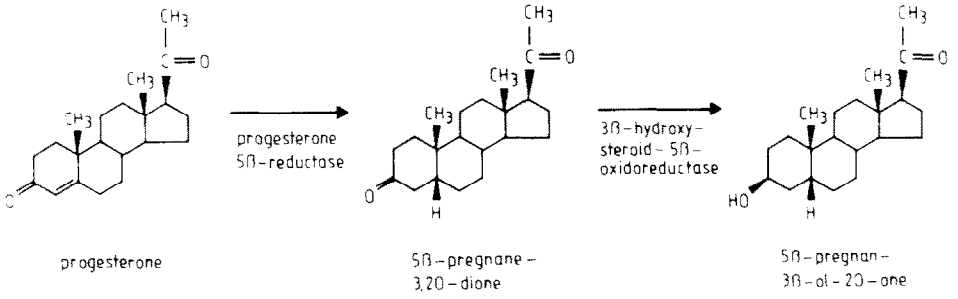

Fig. 1. Reaction catalyzed by progesterone 5β-reductase and the subsequent metabolic step catalyzed by a putative 3β-hydroxysteroid-5β-oxidoreductase.

2.3. *Standard enzyme assay*

The assay contained a total of 1.5–2.5 mg protein, 5 mM glucose 6-phosphate, 1 mM $NADP^+$, 8.4 nkat glucose 6-phosphate dehydrogenase and 40 μM progesterone in buffer at pH 8 (s.a.), yielding a final vol. of 5 ml. After a preincubation period of 20 min the reaction was initiated by the addition of progesterone. The incubation was carried out for 3 h at 30°C. The reaction was terminated by adding 10 ml methylene dichloride while shaking. 5α-Androstan-17β-ol-3-one was used as an internal standard. The extraction of the pregnanes was performed as described previously [5].

2.4. *Product identification and quantification*

Gas-liquid chromatography was performed on a Shimadzu GC-9A with a fused silica capillary column (DB-1701, 30 m, 0.25 mm i.d.). The temperature was 275°C and N_2 was used as the carrier gas. The products were identified by co-chromatography with known standards (all steroids were purchased from Sigma, FRG) and by g.c./m.s. on a Finnigan Mat 112 S using the identical column, but with H_2 as the carrier gas. M.S. 70 eV (rel. int.): 5β-pregnane-3,20-dione: *m/z* 316 (M^+, 8), 298 (M^+-H_2O, 12), 255 (M^+-C_2H_3O, 8), 121 (C_8H_9O, 21), 43 (C_2H_3O, 100).

3. RESULTS

3.1. *Product identification and quantification*

The enzyme activity was found in the soluble fraction (supernatant from 45 000 × *g* and 100 000 × *g* centrifugations) of homogenates from leaf tissue of *Digitalis purpurea*. The product was identified as 5β-pregnane-3,20-dione (Fig. 2). It was clearly separated from its 5α-isomers as was shown by the relative retention times (RRT) of known standards. All amounts of the product formed in vitro were calculated using the relative response factor obtained from standard mixtures of known concentrations and with 5α-androstan-17β-ol-3-one as an internal standard. Further proof for product identification came from g.c./m.s. data (see section 2).

3.2. *Properties of the enzyme*

In the time course (Fig. 3) progesterone 5β-reductase showed a linear reaction for the first 4 h of the incubation period. The following reaction leading to 5β-pregnan-3β-ol-20-one, thought to be part of the putative pathway leading to cardenolides, could also be observed. At longer incubation times the catalytic activity decreases slowly. This decrease may be due to the effects of subsequent reaction steps.

The assay was carried out at different temperatures ranging from 0 to 70°C. The optimum temperature was at 30°C.

The effect of pH was examined with the buffers Bis-Tris and Hepes (see section 2). The optimum activity was achieved at pH 8.0. At pH values exceeding 8 a sharp decrease was observed.

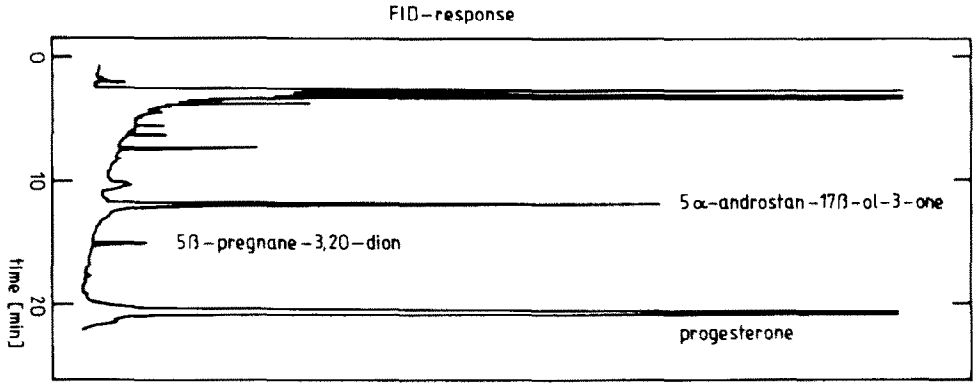

Fig. 2. Gas chromatogram of a standard enzyme assay. The enzymic product 5β-pregnane-3,20-dione (RRT: 1,281), was formed in vitro. Progesterone was used as substrate of the enzymic reaction and 5α-androstan-17β-ol-3-one (100 mM) as internal standard. Further confirmation of product identity came from g.c./m.s data.

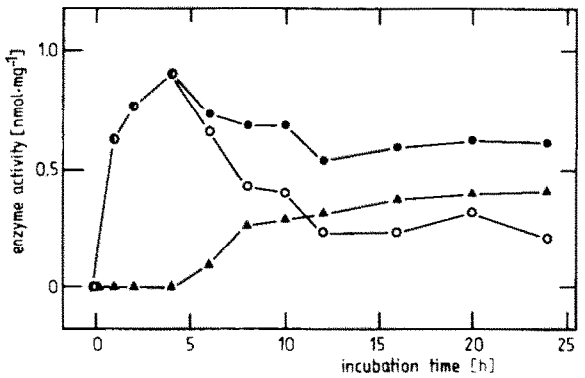

Fig. 3. Time course of the enzymic reaction. Standard enzyme assays were incubated from 0–24 h at 30°C. The products were identified as (○—○) 5β-pregnane-3,20-dione and (▲—▲) 5β-pregnan-3β-ol-20-one. (●—●) Sum of reaction products.

3.3. *Co-factor requirements*

As can be seen from Table I progesterone 5β-reductase requires NADPH as a co-substrate. It cannot be replaced by NADH. An apparent K_m value of 22 μM was detected for NADPH. When NADPH was used as a reduction equivalent the enzyme activity decreased to 1/3 of that found in enzyme assays incubated with the NADPH-regenerating system. The advantage of the NADPH-regenerating system is that there is a constant quantity of reduction equivalents over a long incubation period.

The effect of monovalent and bivalent cations was investigated at concentrations of 0.2 mM and 2 mM. An enzyme assay without cations served as a standard (100% catalytic activity). Enzyme activity could not be stimulated by monovalent cations (Na^+, Li^+, K^+). Bivalent cations (Co^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}) had no effect at lower concentrations, whereas higher concentrations were inhibitory, with the sole exception of $MgCl_2$.

4. DISCUSSION

A soluble enzyme from leaves of *Digitalis purpurea* is described as progesterone 5β-reductase, which catalyzes the reduction of progesterone to 5β-pregnane-3,20-dione. The putative metabolic pathway of cardenolides is based mainly on tracer studies which clearly demonstrated that 5β-pregnane derivatives are precursors in vivo. Thus progesterone 5β-reductase is the first stereospecific enzyme in the putative biosynthetic pathway. It is widely accepted that cholesterol, pregnenolone, progesterone and 5β-dihydro-pregnane derivatives are part of the cardenolide biosynthesis. The enzyme Δ5-3β-hydroxysteroid dehydrogenase/Δ5–Δ4-ketosteroid isomerase (3β-HSD), which catalyzes the conversion of pregnenolone to progesterone, was described as a soluble enzyme in cell cultures of *Digitalis lanata* [7]. Keeping the biosynthetic pathway in mind, which leads to the cardenolides, we can suggest, on the one hand, that the 3β-HSD is part of the putative cardenolide biosynthetic pathway and that, on the other hand, it has a function in the general steroid metabolism of plant cells.

The reduction of progesterone results in two isomeric compounds with 5α- and 5β-configurations. As shown in a previous paper cell cultures of *Digitalis lanata* contain a progesterone 5α-reductase with a definite microsomal localization [5], whereas the enzyme described in the present paper belongs to the soluble fraction of the cell. A quite similar situation was found in the rat liver [4]. The subsequent enzyme, which has been characterized as a 3β-hydroxysteroid-oxidoreductase, forms 5α-pregnan-3β-ol-20-one from 5α-pregnane-3,20-dione. It was isolated from cell-suspension cultures of *Digitalis lanata*. The major part of the catalytic activity turned out to be soluble, whereas a distinctly smaller part seemed to be associated with the endoplasmic reticulum [8].

A parallel reaction leading to the analogous 5β-derivative was observed in the soluble fraction (s.a.) of the cell. Since maximum activities of progesterone 5α-reductase, 3β-hydroxysteroid-oxidoreductase and 5β-HSD were observed in the growth phase of cell cultures, we postulate that these enzyme activities are part of the primary metabolism (general steroid pathway) providing constituents for biomembranes. In order to prove this hypothesis, cell cultures have to be investigated for progesterone 5β-reductase activity. They should show a maximum of activity in the stationary phase of the growth cycle, whereas the progesterone 5α-reductase is active during the growth phase.

Table I

Pyridine nucleotide requirements of progesterone 5β-reductase

Co-substrate added	Relative activity (%)
NADPH regenerating system	100
$NADPH_2$	38.5
$NADH_2$	0

The co-substrates added were 1 mM NADPH, NADH or a NADPH-regenerating system consisting of 5 mM glucose 6-phosphate, 1 mM $NADP^+$ and 8.4 nkat of glucose-6-phosphate dehydrogenase/ml.

Acknowledgements: The authors wish to thank Michael Oberndörfer (Department of Chemistry, Tübingen) for performing the gas chromatographic-mass spectrometric analysis and Professor Dr K. Poralla (Department of Biology, Tübingen), who allowed us to use his Shimadzu GC-9A. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

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