Cytochrome P-450-dependent digitoxin 12β-hydroxylase from cell cultures of *Digitalis lanata*

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Received 7 June 1985

A 12β-hydroxylase which catalyzes the hydroxylation of digitoxin and other cardenolides of the A-series to digoxin and the corresponding cardenolides of the C-series was isolated from suspension cultures of *Digitalis lanata*. The enzyme is located in the microsomal fraction and has a pH optimum at 7.5. The K_m value for β-methyldigitoxin is 7.1 μM. O_2 and $NADPH_2$ are essential for the catalytic activity, with an optimum $NADPH_2$ concentration of 1 mM. Carbon monoxide inhibits the enzyme, but this inhibition is reversible with 450 nm light. This would suggest that the enzyme is a cytochrome P-450-dependent mixed-function monooxygenase. *p*-Hydroxymercuribenzoate and $CuSO_4$ are also inhibitory. KCN stimulates hydroxylation in vitro.

Digitoxin 12β-hydroxylase Digitalis lanata *Biotransformation* *Cytochrome P-450* *Cardiac glycoside*

1. INTRODUCTION

The biotransformation of β-methyldigitoxin has been studied intensively for many years [1]. Until now, though, nothing was known about the enzymes involved in these biotransformation reactions. In animals and microorganisms, the hydroxylation of steroid-like molecules is mostly performed by microsomal cytochrome P-450-dependent monooxygenases. Cytochrome P-450 is also involved in many hydroxylation reactions in higher plants, e.g. cinnamic acid 4-hydroxylase [2], flavonoid 3'-hydroxylase [3], or monoterpene hydroxylase [4,5], and some other systems. Here we describe a new cytochrome P-450-dependent hydroxylase which catalyzes the 12β-hydroxylation of digitoxin to digoxin in cell cultures of *Digitalis lanata*.

2. MATERIALS AND METHODS

2.1. *Cell suspension cultures*

Cell cultures of *D. lanata* were the generous gift of Professor E. Reinhard, Tübingen. They were cultivated by adding 10 ml of an 8-day-old suspension to 50 ml fresh medium every 8 days [1]. The cells harvested for experimental use were 5–7 days old.

2.2. *Chemicals*

β-Methyldigoxin was purchased from Roth (Karlsruhe), digitoxin and digoxin from Serva (Heidelberg). All other cardiac glycosides were gifts from Dr Alfermann, Tübingen.

2.3. *Preparation of microsomes*

Cell suspensions were filtered under suction. The cell material was ground in a mortar together with sand and 1 ml buffer (0.1 M NaH_2PO_4/Na_2HPO_4, 1 mM EDTA, 0.6 M mannitol, 14 mM mercaptoethanol, pH 7.5) per g fresh wt. The homogenate was filtered through Miracloth and centrifuged for 20 min at $8000 \times g$. The supernatant was stirred with 50 μl of 1 M $MgCl_2$ per ml for 20 min and then centrifuged at $49000 \times g$ for 20 min. The supernatant was then discarded and the pellet resuspended in buffer to a final protein concentration of 0.6 mg/ml. Protein concentrations were determined according to Bradford [6]. In some experiments the microsomal fraction was

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

prepared by centrifuging the $8000 \times g$ supernatant at $100000 \times g$ for 1 h.

2.4. *Enzyme assay*

The assay consisted of 3 mg microsomal protein, 7.5 mM glucose 6-phosphate, 2.5 U glucose-6-phosphate dehydrogenase, 1 mM $NADP^+$ and 50 μM β-methyldigitoxin in 80% methanol in a final volume of 5 ml and at pH 7.5 (final methanol concentration 0.2%). The assay was incubated at 26°C in 25 ml Erlenmeyer flasks for 4 h. The reaction was terminated by shaking with 10 ml chloroform and freezing.

The cardiac glycosides were extracted by shaking the total assay first with 10 ml, then with another 5 ml chloroform. The collected organic phases were evaporated under vacuum, redissolved in 1.2 ml chloroform, transferred to Eppendorf vials and dried again. The samples were washed twice with 0.5 ml petroleum benzine (40–60°C). After sedimentation at $16000 \times g$ for 5 min the pellet was dissolved in 25 μl methanol (80%).

2.5. *HPLC and TLC analysis of the reaction product*

HPLC was performed on a Hypersil ODS column (25 cm, particle size 5 μm) with pumps and integrator from Spectraphysics (Darmstadt). The cardiac glycosides were detected at 220 nm after isocratic elution with an 80% methanol/20% H_2O eluent (flow rate 1 ml/min). For identification, the retention times of the cardiac glycosides in the assay were compared to the retention times of authentic reference substances. In addition, TLC was carried out on reversed-phase RP-18 plates (Merck, Darmstadt) in 60% acetone/40% H_2O. The cardiac glycosides were detected by spraying with trichloroacetic acid/chloramine T according to Kaiser [7].

2.6. *Experiments with different gas mixtures*

The gas mixtures were produced by displacement of water in a calibrated separation funnel using commercially available pure gases. The reaction mixtures without β-methyldigitoxin were placed into vacuum tubes, which were evacuated for 1 min and then filled with the gas mixture for 30 s a total of 3 times. β-Methyldigitoxin was added with a syringe through the silicone stopper in order to start the reaction. The blue light treatment was performed using a slide projector and a filter (λ_{Tmax} 450 nm).

3. RESULTS

3.1. *Product identification*

The hydroxylated products of β-methyldigitoxin and digitoxin were identified as β-methyldigoxin and digoxin, respectively, by comparing the retention times from HPLC and the R_f values and UV fluorescence from TLC after spraying with trichloroacetic acid/chloramine T with those of authentic reference substances. This shows that hydroxylation occurs at position 12 of the steran. Fig.1 depicts a HPL chromatogram.

3.2. *Properties of the enzyme*

Molecular oxygen is essential for enzyme activity. After treatment with nitrogen gas no hydroxylation product could be found (table 1). Thiol reagents stimulated 12β-hydroxylase activity at low

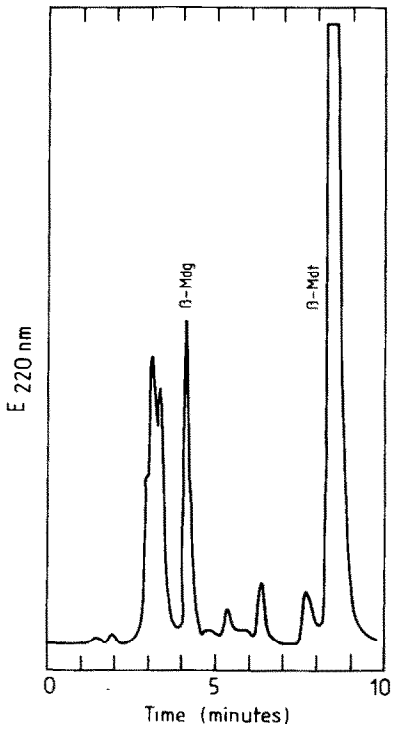

Fig.1. HPL chromatogram of a 12β-hydroxylase assay prepared for HPLC as described in section 2. β-Mdg, β-methyldigoxin, β-Mdt, β-methyldigitoxin.

Table 1

Effect of different gas phase compositions on 12β-hydroxylase activity.

Gas phase	Activity (%)
Air	100
100% N_2	10
15% O_2 + 85% N_2	117
15% O_2 + 15% CO + 70% N_2	60
15% O_2 + 15% CO + 70% N_2 + 450 nm light	98
5% O_2 + 5% CO + 90% N_2	30

For production of gas phases and treatment and composition of test assays see section 2. 100% = 124.3 nkat/kg

concentrations, but higher concentrations were inhibitory (table 2). Most effective was DTT, which caused a product increase of 34%. The sulfhydryl reagent *p*-hydroxymercuribenzoate inhibited activity totally at a concentration of 0.1 mM, whereas KCN, which is a typical inhibitor for heme enzymes except for cytochrome P-450, increased 12β-hydroxylase activity by 23% at concentrations of 0.1 and 1 mM. EDTA was slightly stimulating for the enzyme with an optimum at 1 mM.

The optimum pH value for the hydroxylation of β-methyldigitoxin was 7.5, with half-maximum activities at pH 6.5 and 8.4. Hydroxylation in vitro proceeds linearly up to 4 h, after which the reaction rate decreases slowly. The enzyme can be stored for several days at −18°C in the presence of 15% glycerol; it then retains 70% of its original activity.

Table 2

Effect of thiol reagents and inhibitors on 12β-hydroxylase activity

Addition	Concentration (mM)	Relative activity (%)
Mercaptoethanol	0	100
	1.4	105
	14	93
	28	86
Dithiothreitol	1	134
	10	85
Glutathione	1	118
	10	88
$CuSO_4$	0.1	94
	1	5
KCN	0.1	122
	1	123
p-Hydroxymercuribenzoate	0.5	6

3.3. *Subcellular localization*

The 12β-hydroxylase from *D. lanata* cell cultures is located in the microsomal fraction (100 000 × *g* pellet or $MgCl_2$ precipitate). No activity was found in the 8000 × *g* pellet. In the 100 000 × *g* supernatant only 10% of the total activity can be found.

3.4. *Cofactor requirements*

The 12β-hydroxylase requires $NADPH_2$ and was saturated at a concentration of 1 mM. In the standard assay $NADPH_2$ was supplied by a regenerating system consisting of glucose 6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$, which increased hydroxylase activity by 18% (table 3). $NADP^+$ alone or $NADH_2$ could not replace $NADPH_2$. No synergistic effect could be observed if $NADPH_2$ and $NADH_2$ were applied together. Furthermore, the addition of $NADP^+$ and $NADH_2$ with or without ATP does not lead to the hydroxylation of β-methyldigitoxin. This demonstrates that no transhydrogenase is present in the microsomal fraction. The addition of FAD or FMN alone showed no significant hydroxylase activity, but simultaneous addition to the $NADPH_2$-regenerating system led to an increase in activity. This effect was highest when FAD and FMN were added together.

3.5. *Substrate specificity*

In keeping with the biotransformation substrate in vivo [1], β-methyldigitoxin was used as a substrate for the 12β-hydroxylase. Other cardenolides such as acetyldigitoxin, digitoxin and digitoxigenin were also hydroxylated. The substrates were added in 80% methanol; the final concentration of methanol in the assay mixture of 0.2% did not influence enzyme activity. The optimum β-methyldigitoxin concentration was 20 μM. At higher concentrations slight substrate inhibition was observed. The K_m value for β-methyldigitoxin, calculated frrom Lineweaver-Burk plots, was 7.1 μM.

Table 3

Cofactor requirement of 12β-hydroxylase

	Addition	Relative activity (%)
(a,b)	1 mM $NADP^+$ + RS	100
(a)	1 mM $NADPH_2$	92
(a)	0.5 mM $NADPH_2$	86
(a)	1 mM $NADH_2$	18
(a)	0.5 mM $NADH_2$	0
(a)	1 mM $NADP^+$	0
(a)	0.5 mM $NADP^+$	0
(a)	0.5 mM $NADPH_2$ + 0.5 mM $NADH_2$	85
(a)	0.5 mM $NADP^+$ + 0.5 mM $NADH_2$	14
(a)	0.5 mM $NADP^+$ + 0.5 mM $NADH_2$ + 0.1 mM ATP	19
(b)	5 μM FAD	19
(b)	5 μM FMN	11
(b)	1 mM $NADP^+$ + RS + 5 μM FAD	32
(b)	1 mM $NADP^+$ + RS + 5 μM FMN	128
(b)	1 mM $NADP^+$ + RS + 5 μM FAD + 5 μM FMN	148

The test assays consisted of approx. 3 mg microsomal protein in buffer, 50 μM β-methyldigitoxin and the indicated additions in a final volume of 5 ml. RS, $NADPH_2$-regenerating system (see section 2). The specific hydroxylase activities are (a) 301 and (b) 154 nkat/kg

3.6. *Effect of carbon monoxide*

Microsomal fractions were incubated under controlled gas mixtures containing N_2, O_2 and CO (table 1). After treatment with pure N_2 only 10% hydroxylase activity could be observed, showing that O_2 is essential for hydroxylation. In an atmosphere containing 5% O_2 and 5% CO the inhibition by CO was 70%. In an atmosphere composed of 15% O_2 and 15% CO the enzyme was inhibited by 40%. Illumination with blue light (λ_{Tmax} 450 nm) reversed this inhibition almost totally.

4. DISCUSSION

This paper describes a 12β-hydroxylase involved in cardiac glycoside biotransformation by cell cultures of *D. lanata*. The enzyme catalyzes the hydroxylation of digitoxin and other cardenolides of the A-series. Activity is located in the microsomal fraction. The enzyme needs O_2 and $NADPH_2$. The inhibition of hydroxylation by CO and the reversibility of this inhibition with 450 nm light as well as the stimulation of the reaction by KCN suggests the involvement of cytochrome P-450, which is present in *Digitalis* cells (Petersen, unpublished). Since the reaction is strongly inhibited by *p*-hydroxymercuribenzoate and stimulated by thiol reagents, the participation of sulfhydryl groups is very likely. It remains to be shown that the 12β-hydroxylase is also present in intact *D. lanata* plants and in the different cell strains which carry out biotransformations in vivo such as glucosylation and demethylation [1]. The subcellular localization also remains to be investigated in detail using linear sucrose gradients.

ACKNOWLEDGEMENTS

We wish to thank Professor E. Reinhard and Dr A. Alfermann for their support of this work. This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

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