Reconstitution of cytochrome *P*-450-dependent digitoxin 12 β -hydroxylase from cell cultures of foxglove (*Digitalis lanata* EHRH.)

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Cytochrome *P*-450-dependent digitoxin 12 β -hydroxylase from cell cultures of foxglove (*Digitalis lanata*) was solubilized from microsomal membranes with CHAPS {3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonic acid}. Cytochrome *P*-450 was separated from NADPH:cytochrome *c* (*P*-450) reductase by ion-exchange chromatography on DEAE-Sephacel. NADPH:cytochrome *c* (*P*-450) reductase was further purified by affinity chromatography on 2',5'-ADP-Sepharose 4B. This procedure resulted in a 248-fold purification of the enzyme; on SDS/polyacrylamide-gel electrophoresis after silver staining, only one band, corresponding to a molecular mass of 80 kDa, was present. The digitoxin 12 β -hydroxylase activity could be reconstituted by incubating partially purified cytochrome *P*-450 and NADPH:cytochrome *c* (*P*-450) reductase together with naturally occurring microsomal lipids and flavin nucleotides. This procedure yielded about 10% of the original amount of digitoxin 12 β -hydroxylase.

INTRODUCTION

The biotransformation of β -methyl digitoxin to β -methyl digoxin (Scheme 1) has been extensively studied in cell cultures of foxglove (*Digitalis lanata*) (Alfermann *et al.*, 1977). This metabolic step is mediated by a microsomal mixed-function cytochrome *P*-450-dependent mono-oxygenase which is located in the endoplasmic reticulum. This enzyme was first detected in *Digitalis lanata* cell cultures (Petersen & Seitz, 1985).

Cytochrome *P*-450-dependent hydroxylations are well investigated in animals and prokaryotes, and, with regard to plant cells, the number of examples of this type of enzymic catalysis is increasing, but the reconstitution of

a cytochrome *P*-450-dependent enzyme by partially purified constituents has been carried out successfully in only two cases: cinnamic acid 4-hydroxylase from Jerusalem artichoke (*Helianthus tuberosus*) (Benveniste *et al.*, 1986; Gabriac *et al.*, 1985) and a monoterpene hydroxylase from the Madagascar periwinkle (*Catharanthus roseus*) (Madyastha *et al.*, 1976; Madyastha & Coscia, 1979). Cytochrome *P*-450-dependent metabolic pathways in plant cells were described for the first time in gibberellin biosynthesis (Murphy & West, 1969). Since then, many cytochrome *P*-450-dependent enzymes from higher plants, mostly involved in secondary metabolism, were described, for example flavonoid 3'-hydroxylase (Hagmann *et al.*, 1983; Larson & Bussard, 1986), ferulic acid 5-hydroxylase (Grand, 1984), ipomeamarone 15-hydroxylase (Fujita *et al.*, 1982), 3,9-dihydroxypterocarpane 6a-hydroxylase (Hagmann *et al.*, 1984) and 5-*O*-(4-coumaroyl)shikimate 3'-hydroxylase (Heller & Kühnl, 1985).

In the present paper the purification of stable constituents of the digitoxin 12 β -hydroxylase from *Digitalis lanata* cell cultures is described. First the proteins cytochrome *P*-450 and NADPH:cytochrome *c* (*P*-450) reductase were solubilized from the microsomal membrane. After the separation and purification of the proteins by ion-exchange and affinity chromatography, the digitoxin 12 β -hydroxylase was reconstituted from the partially purified components *in vitro*.

MATERIALS AND METHODS

Materials

Cell cultures of *Digitalis lanata* were cultivated as described previously (Petersen & Seitz, 1985). β -Methyl-

Scheme 1. Reaction catalysed by digitoxin 12 β -hydroxylase with β -methyl digitoxin as substrate

Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonic acid; DTT, DL-dithiothreitol; PAGE, polyacrylamide-gel electrophoresis.

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digitoxin was a gift from Dr. A. W. Alfermann (University of Tübingen). β -Methyldigoxin was obtained from Roth (Karlsruhe, Germany). The detergents CHAPS, digitonin, Triton X-100 and Tween 80 were from Serva (Heidelberg, Germany), sodium cholate and *n*-octyl glucoside were from Merck (Darmstadt, Germany), and DEAE-Sephacel and 2',5'-ADP-Sephrose 4B were obtained from Pharmacia (Uppsala, Sweden).

Enzyme preparation

All steps were performed at 0–4 °C. Suspension cells of *Digitalis lanata*, 4–6 days old, were harvested by filtration under suction. They were ground in a precooled mortar together with quartz sand and 1 ml of buffer [0.1 M-Tris/HCl (pH 7.5)/0.6 M-mannitol/1 mM-DTT/1 mM-EDTA]/g fresh weight. The homogenate was filtered through Miracloth and centrifuged at 8000 *g* for 20 min. A 1 M-MgCl₂ solution was added to the supernatant up to a final concentration of 50 mM. This preparation was stirred for 20 min. After a 20 min run at 48000 *g*, the microsomes were resuspended in 0.1 M-Tris/HCl (pH 8.0)/0.6 M-mannitol/1 mM-DTT/1 mM-EDTA/15 % (v/v) glycerol.

Solubilization

The microsomal suspension, with a final protein concentration of 2.5 mg · ml⁻¹, was solubilized by adding a 10 % (w/v) CHAPS solution dropwise up to a final CHAPS concentration of 1 %. The assay mixture was stirred on ice for 30 min and then centrifuged at 100000 *g* for 1 h. The supernatant was used for further purification steps.

Ion-exchange chromatography

Precycled DEAE-Sephacel was equilibrated in 0.1 M-Tris/HCl (pH 8.0)/0.1 mM-EDTA/1 mM-DTT/15 % (v/v) glycerol/0.06 % CHAPS. In some experiments the buffer contained also 0.1 % Tween 80. A portion (5 mg) of the solubilized microsomal protein was applied to a column (1.7 cm × 7.5 cm) and eluted with a KCl step gradient consisting of 100 ml of buffer without KCl and 100 ml each of 0.2 M-, 0.3 M- and 0.5 M-KCl in buffer. The elution medium was pumped by a peristaltic pump (Ismatec, mp-ge, Zürich, Switzerland) at a flow rate of 20 ml · h⁻¹. Fractions (10 ml each) were collected. Fractions containing cytochrome *P*-450 or NADPH:cytochrome *c* (*P*-450) reductase activity were concentrated separately by ultrafiltration and stored at -18 °C.

Affinity chromatography

2',5'-ADP-Sephrose 4B was filled into a column (1.9 cm × 6 cm). The concentrated fractions containing NADPH:cytochrome *c* (*P*-450) reductase were applied to the column and eluted with 40 ml of 0.01 M-Na₂HPO₄/NaH₂PO₄ (pH 7.6)/15 % glycerol (v/v)/0.06 % CHAPS/0.02 mM-EDTA/0.2 mM-DTT (equilibration buffer), 60 ml of 0.2 M-Na₂HPO₄/NaH₂PO₄ (pH 7.6)/15 % glycerol/0.06 % CHAPS/0.2 mM-DTT/0.4 mM-EDTA, 20 ml of equilibration buffer and 60 ml of a linear gradient of 0–5 mM-2'-AMP in equilibration buffer. The flow rate was adjusted to 20 ml · h⁻¹. Fractions (10 ml each) were collected. The fractions containing NADPH:cytochrome *c* (*P*-450) reductase activity were combined, concentrated by ultrafiltration and stored at -18 °C.

Determination of cytochrome *P*-450 and cytochrome *b*₅

The cytochrome *P*-450 content was calculated from the CO-difference spectra of sodium dithionite-reduced fractions according to Omura & Sato (1964) in a Uvicon 810 spectrophotometer (Kontron, Eching, Germany). The cytochrome *b*₅ content was determined using the difference spectra of oxidized and NADH-reduced fractions according to Estabrook & Werringloer (1978).

Determination of NADPH:cytochrome *c* (*P*-450) reductase activity

The assay for NADPH:cytochrome *c* (*P*-450) reductase was modified from Madyastha *et al.* (1976). The NADPH-dependent reduction of cytochrome *c* was measured spectrophotometrically at 550 nm. The assay mixture contained 1.78–1.87 ml of 0.5 M-Tris/HCl, pH 7.6, 20 μ l of 50 mM-KCN, 50 μ l of 2 mM-cytochrome *c* and 10–100 μ l of the enzyme preparation in a total volume of 2 ml. The increase in absorption was observed for a few minutes, then 50 μ l of 6 mM-NADPH was added and the absorption was measured again. When testing purified fractions, 10 μ l each of 1 mM-FAD and 1 mM-FMN were added to reconstitute the reductase. The enzyme activity was calculated using a millimolar absorption coefficient of 21 mM⁻¹ · cm⁻¹.

Digitoxin 12 β -hydroxylase activity

In essence, the digitoxin 12 β -hydroxylase activities of microsomes (microsomal fractions) and of solubilized and reconstituted fractions were determined as described previously (Petersen & Seitz, 1985).

Isolation and identification of microsomal lipids

Microsomal lipids were extracted from microsomes of *Digitalis lanata* by treatment with chloroform/methanol (2:1, v/v). Any protein was removed by washing the organic phase with 0.9 % NaCl solution. For reconstitution experiments the total microsomal lipids were suspended in buffer [0.1 M-Tris/HCl (pH 7.5)/1 mM-DTT/1 mM-EDTA/0.6 M-mannitol] by sonication. The phospholipids were enriched by the method described by Kates (1972). They were analysed by two-dimensional t.l.c. in chloroform/methanol/water (65:25:4, by vol.) and chloroform/methanol/25 % NH₃ (65:35:4, by vol.) (Osagie & Kates, 1984) and made visible with a specific spray (Vaskowsky & Kostetsky, 1968). The *R_F* values were compared with those of authentic phospholipids.

Reconstitution experiments

Reconstitution assays contained, per ml, 56 pmol of partially purified cytochrome *P*-450, 16.7 nkat of NADPH:cytochrome *c* (*P*-450) reductase purified by affinity chromatography, 0.25 mg of sonicated microsomal lipids and 10 nmol each of FAD and FMN. The above-mentioned components were incubated for 15 min at room temperature, then the substrates for a digitoxin 12 β -hydroxylase standard assay, 20 μ M- β -methyldigitoxin and 1 mM-NADPH supplied by the regenerating system, were added. The mixture was incubated for 4 h at 26 °C in a total volume of 5 ml. The cardiac glycosides were extracted and analysed by the standard procedure described previously (Petersen & Seitz, 1985).

Table 1. Effect of different detergents on digitoxin 12 β -hydroxylase activity

All samples contained 15% (v/v) glycerol, except the control.

Treatment	Detergent/ protein ratio	Relative digitoxin 12 β -hydroxylase activity (%)
Control	—	202
Control + glycerol	—	100
Triton X-100	0.2:1	83
	0.4:1	78
Tween 80	0.2:1	120
	0.4:1	119
Digitonin	0.2:1	104
	0.4:1	100
Sodium cholate	0.2:1	104
	0.4:1	97
Octyl glucoside	0.2:1	98
	0.4:1	122

SDS/PAGE

SDS/PAGE of the purified NADPH:cytochrome *c* (*P*-450) reductase was performed essentially as described by Laemmli (1970).

Protein determination

Protein concentrations were measured by the method of Bradford (1976), with bovine serum albumin as a standard.

RESULTS

Solubilization of microsomal proteins

In order to separate and purify the constituents of the cytochrome *P*-450-dependent digitoxin 12 β -hydroxylase, the membrane-bound proteins need to be solubilized. Several detergents were tested for their influence on digitoxin 12 β -hydroxylase activity at different detergent-to-protein ratios. The results are summarized in Table 1. Treatment with Triton X-100 leads to the lowest digitoxin 12 β -hydroxylase activity. Octyl glucoside, Tween 80 and digitonin did not affect the enzyme activity or were slightly stimulatory. Among the compounds tested, Tween 80 seemed to be the best-suited for solubilizing the enzyme. Tween 80 concentrations exceeding 0.5 μ g/mg of protein resulted in the slight decrease in digitoxin 12 β -hydroxylase activity, which suggests that cytochrome *P*-450 and NADPH:cytochrome *c* (*P*-450) reductase might be distributed into different micella, resulting in a loss of the hydroxylase activity. A very similar situation was found with CHAPS. This zwitterionic detergent with a very low critical micellar concentration was found to be best suited for the solubilization of the constituents of digitoxin 12 β -hydroxylase. It does not interfere with Bradford's (1976) protein determination method. With 0.1–1% CHAPS, both constituents of the enzyme also seem to be distributed to different micella. During standard solubilization procedures with CHAPS, a detergent concentration of 1% was used. It resulted in a complete solubilization of the NADPH:cytochrome

Fig. 1. DEAE-Sephacel chromatography of microsomal proteins solubilized with 1% CHAPS

Proteins were eluted with a step gradient of KCl in 0.1 M-Tris/HCl, pH 8, with 0.1 mM-EDTA, 1 mM-DTT, 15% glycerol and 0.06% CHAPS. Fractions (10 ml each) were collected and assayed for cytochrome *P*-450 and NADPH:cytochrome *c* (*P*-450) reductase activity. NADPH:cytochrome *c* (*P*-450) reductase was assayed in the presence (or absence) of 5 μ M-FAD and -FMN.

c (*P*-450) reductase activity and of 74% of the cytochrome *P*-450.

Separation and purification of cytochrome *P*-450 and NADPH:cytochrome *c* (*P*-450) reductase

Plant cytochrome *P*450-dependent enzymes seem to be composed of at least three constituents: cytochrome *P*-450, NADPH:cytochrome *c* (*P*-450) reductase and microsomal lipids. In animal cells, additional constituents have been detected, such as adrenodoxine in the mitochondria of the adrenal cortex, and cytochrome *b*₅ and the corresponding reductase in hepatic microsomes having mono-oxygenase activity. The two plant enzymes investigated up to now in detail were a monoterpene hydroxylase from *Catharanthus roseus* (Madyastha *et al.*, 1976; Madyastha & Coscia, 1979) and a cinnamic acid 4-hydroxylase from *Helianthus tuberosus* (Gabriac *et al.*, 1986; Benveniste *et al.*, 1986). A NADPH:cytochrome *c* reductase was investigated by Fujita & Asahi (1985), and a cytochrome *P*-450 of unknown function was purified from tulip (*Tulipa gesneriana*) bulbs by Higashi *et al.* (1983, 1985). The purification procedure for digitoxin 12 β -hydroxylase followed the protocol described by Madyastha & Coscia (1979) with several modifications.

Ion-exchange chromatography

Digitoxin 12 β -hydroxylase was solubilized with 1% CHAPS from the microsomal membranes. The 100000 g supernatant was layered on a DEAE-Sephacel column and the protein was eluted with a step gradient having steps of 0 M-, 0.2 M-, 0.3 M- and 0.5 M-KCl. Cytochrome *P*-450 and NADPH:cytochrome *c* (*P*-450) reductase were well separated (Fig. 1). The cytochrome *P*-450 was eluted in KCl-free elution buffer, whereas the reductase peaked at 0.2 M-KCl. The cytochrome *P*-450-enriched fractions contained no cytochrome *b*₅ and only traces of NADPH:cytochrome *c* (*P*-450) reductase activity. The peak fraction of NADPH:cytochrome *c* (*P*-450) reductase contained only minor traces of cytochrome *P*-450. The reductase activity eluted from the

Table 2. Purification protocol for NADPH:cytochrome *c* (P-450) reductase

The microsomal proteins were solubilized with 1% CHAPS. The elution buffer for both chromatographic steps contained 0.1% Tween 80. NADPH:cytochrome *c* (P-450) reductase was assayed in the absence (–F) and the presence (+F) of flavin nucleotides.

Purification step	Total protein (mg)	Enzyme activity (nkat)	Specific activity (mkat/kg)	Purification (-fold)	Yield (%)
Microsomes	11.1	–F 369 +F 730	26.5 52.2	–	100
Solubilized microsomes	5.0	–F 374 +F 526	74.6 104.8	2.8	101
DEAE-Sephacel column	2.6	–F 340 +F 617	96.8 211.6	3.7	92
Affinity chromatography (2',5'-ADP-Sepharose 4B)	0.03	–F 171 +F 455	1467.0 12940.0	55.0 248.0	46 52

Table 3. Purification protocol for cytochrome (Cyt.) P-450

The protein was solubilized with 1% CHAPS

Purification step	Total protein (mg)	Cyt. P-450 (pmol)	Cyt. P-450 (pmol/mg)	Purification (-fold)	Yield (%)
Microsomes	9.5	1070	113	–	100
Solubilized microsomes	5.0	795	159	1.4	74
DEAE-Sephacel column	0.4	287	683	6.0	27

column seemed rather low, but could be clearly stimulated by adding flavins to the assay. In standard assays, 5 μ M-FAD and 5 μ M-FMN were present. This stimulation of the NADPH:cytochrome *c* (P-450) reductase by the addition of flavins is explained by the fact that the reductase is a flavoprotein and loses some of its prosthetic flavin groups during the purification procedure; these can, however, be replaced by exogenous FAD and FMN. This was also reported for the purification of NADPH:cytochrome *c* (P-450) reductase from other sources. The addition of FMN was more effective than the addition of FAD. The reductase was saturated at a concentration of 5 μ M-FMN, but not until 15 μ M-FAD was used.

With this protocol, an insufficient purification of the NADPH:cytochrome *c* (P-450) reductase was obtained, and the yield was rather low, namely 48%, in spite of the addition of flavins. The addition of 0.1% Tween 80, however, resulted in a 225% increase in NADPH:cytochrome *c* (P-450) reductase activity, resulting in a purification of 4.4-fold and a yield of 85% (Table 2). Therefore 0.1% Tween 80 was added to the elution media. The elution pattern was not affected by Tween 80. The stimulation of the reductase activity is due only to Tween 80, since the same results were obtained regardless of whether the detergent was added to the elution buffer

or to the NADPH:cytochrome *c* (P-450) reductase assay mixture.

With regard to cytochrome P-450 (Table 3), the purification was 6-fold and the yield was nearly 30%. The low yield must be due to the transformation of cytochrome P-450 into its degradation product, cytochrome P-420. This can be shown by spectral data.

For further experiments the fractions containing cytochrome P-450 or NADPH:cytochrome *c* (P-450) reductase were concentrated separately by ultrafiltration and stored at –18 °C.

Affinity chromatography with 2',5'-ADP-Sepharose 4B

Yasukochi & Masters (1976) used 2',5'-ADP-Sepharose 4B in order to purify NADPH:cytochrome *c* reductase from rat and pig livers. The NADPH-binding site of the enzyme interacts specifically with 2',5'-ADP bound to the matrix of the column and can be eluted with a linear gradient of 2'-AMP. The same procedure was used to purify further the NADPH:cytochrome *c* (P-450) reductase from *Digitalis lanata*. The enzyme was bound to the affinity resin and, after washing of the column, was eluted with a linear gradient of 2'-AMP (Fig. 2). The peak fraction of the reductase appeared at a 2'-AMP concentration of 3.6 mM. In this case, also, a stimulation of the reductase activity even higher than that after ion-exchange chromatography was observed with flavin nucleotides. The activity was increased nearly 9-fold. The whole purification protocol is summarized in Table 2. The purification after affinity chromatography was 248-fold and the yield was also very good, namely 62%. At this purification step the addition of 0.1% Tween 80 also stimulated the activity of the reductase as described above for ion-exchange chromatography. FAD and FMN (5 μ M) raised the measurable purification for NADPH:cytochrome *c* (P-450) reductase from 55- to 248-fold and the yield from 48 to 62%, as can be seen from Table 2. For further experiments the fractions from the affinity column containing NADPH:cytochrome *c* (P-450) reductase activity were combined, concentrated by ultrafiltration, and frozen at –18 °C. On SDS/PAGE after silver staining, only one protein band was visible. Its molecular mass was determined to be 80 kDa, compared with 78 kDa for its counterpart in *Catharanthus roseus* (Madyastha & Coscia, 1979), 82 kDa for that in *Helianthus tuberosus* (Benveniste *et al.*, 1986) and 81 kDa for that in the sweet potato (*Ipomoea batatas*) (Fujita & Asahi, 1985).

Identification of microsomal lipids

To reconstitute the digitoxin 12 β -hydroxylase activity, a lipid matrix for the integration of the protein components is necessary. It seems logical to use the naturally occurring lipids of *Digitalis lanata* microsomes. The lipids were extracted from the microsomal pellet with chloroform/methanol. Two-dimensional t.l.c. revealed phosphatidylethanolamine and phosphatidylcholine to be the major phospholipid constituents of the microsomal membranes. Minor components were phosphatidylserine, phosphatidylinositol and phosphatidylglycerol.

Enzyme-substrate interactions of NADPH:cytochrome *c* (P-450) reductase

The substrate specificity of digitoxin 12 β -hydroxylase with regard to co-factor requirements has already been

Fig. 2. Affinity chromatography of NADPH:cytochrome *c* (*P*-450) reductase on a 2',5'-ADP-Sepharose-4B column

Elution was performed with a linear gradient (see inset) of 2'-AMP ranging from 0 to 5 mM. Fractions 0–5, equilibration buffer (0.01 M-sodium phosphate buffer, pH 7.6, containing 0.06% CHAPS, 15% glycerol, 0.2 mM-DTT and 0.02 mM-EDTA. Fractions 6–9, 0.2 M-sodium phosphate buffer, pH 7.6, containing 0.06% CHAPS, 15% glycerol, 0.2 mM-DTT and 0.4 mM-EDTA. Fractions 12–17, linear gradient of 2'-AMP in equilibration buffer (see above). Fractions (10 ml each) were collected and assayed for NADPH:cytochrome *c* (*P*-450) reductase activity in the absence (○) or presence (●) of 5 μ M-FAD and 5 μ M-FMN.

Fig. 3. Lineweaver-Burk plot for purified NADPH:cytochrome *c* (*P*-450) reductase with cytochrome *c* as an electron acceptor

The assay mixture contained 5 μ M-FAD and 5 μ M-FMN. A K_m value of 7.7 μ M was determined for cytochrome *c*.

described (Petersen & Seitz, 1985). The enzyme requires NADPH₂. NADH₂ could not replace NADPH₂. In order to get information on the characteristics of the purified NADPH:cytochrome *c* (*P*-450) reductase experiments were designed to test cytochrome and NADPH-dependency. In Fig. 3 a Lineweaver-Burk plot of the NADPH:cytochrome *c* (*P*-450) reductase with

Fig. 4. Lineweaver-Burk plot for purified NADPH:cytochrome *c* (*P*-450) reductase with increasing concentrations of NADPH

A K_m value of 15.2 μ M was determined.

increasing concentrations of cytochrome *c* is shown. The K_m value for cytochrome *c* was determined to be 7.7 μ M. The same result was obtained with a Hanes plot. This value agrees closely with data obtained for other plant-derived cytochrome *P*-450-dependent enzymes. One has to bear in mind that cytochrome *c* is not the naturally occurring electron donor, since it is not present in endoplasmic-reticular membranes.

In Fig. 4 the NADPH₂-concentration-dependency is depicted in another Lineweaver-Burk plot. The reductase activity was assayed in the presence of flavin nucleotides. The K_m value was calculated to be 15.2 μ M. This is very similar to the value found for the K_m of other plant enzymes of this type. Interestingly, it is very close to the K_m value for the digitoxin 12 β -hydroxylase tested as a whole in microsomes. It was 26 μ M for NADPH₂. This close agreement is a very good indication that the reductase is kept in an intact state during purification.

Reconstitution of digitoxin 12 β -hydroxylase activity

Up to now, only two cytochrome *P*-450-dependent enzymes have been reconstituted from plant-derived constituents *in vitro*. In *Catharanthus roseus* a monoterpene hydroxylase was successfully recombined (Madyastha *et al.*, 1976; Madyastha & Coscia, 1979), and in *Helianthus tuberosus* the cinnamic acid 4-hydroxylase was reconstituted (Gabriac *et al.*, 1985; Benveniste *et al.*, 1986). The reconstitution of digitoxin 12 β -hydroxylase is summarized in Table 4. Cytochrome *P*-450 and NADPH:cytochrome *c* (*P*-450) reductase were incubated at room temperature together with microsomal lipids extracted from *Digitalis lanata* membranes and sonicated in buffer. FAD and FMN were also present. After an incubation period of 15 min, β -methyldigitoxin and a NADPH-regenerating system were added. The incubation took place under standard conditions, the reaction was terminated as described above, and the cardenolides were extracted in the usual way. The product was determined by h.p.l.c. analysis. There was significant hydroxylation of β -methyldigitoxin to β -methyldigoxin, which accounted for 10% of the activity determined in isolated microsomes. In order to reconstitute successfully

Table 4. Reconstitution of digitoxin 12 β -hydroxylase

Partially purified cytochrome *P*-450 (56 pmol·ml⁻¹), NADPH:cytochrome *c* (*P*-450) reductase (16.7 nkat·ml⁻¹) (purified to homogeneity and assayed in the presence of FAD and FMN), microsomal lipids (0.5 mg·ml⁻¹) (suspended by ultrasonication in 0.1 M-Tris/HCl, pH 7.5, containing 0.6 M-mannitol, 1 mM-DTT and 1 mM-EDTA), 10 μ M-FAD, and 10 μ M-FMN were incubated at room temperature for 15 min, then 5 mM-glucose 6-phosphate, 1 mM-NADP⁺, glucose-6-phosphate dehydrogenase (8.4 nkat·ml⁻¹) and 20 μ M- β -methyl-digitoxin were added. The reaction was allowed to proceed in a standard assay mixture (5 ml, 26 °C, 4 h). Cardiac glycosides were extracted and analysed by standard procedures.

Components	β -Methyl-digitoxin (pmol)
Cytochrome <i>P</i> -450	320
NADPH:cytochrome <i>c</i> (<i>P</i> -450) reductase	200
Microsomal lipids	160
Cytochrome <i>P</i> -450 + reductase + lipids	1080

the whole enzyme, the NADPH:cytochrome *c* (*P*-450) reductase had to be purified in the absence of Tween 80, since contamination of the protein with traces of the detergent resulted in a complete loss of activity in the reconstitution *in vitro*. The binding of the substrate to cytochrome *P*-450 and the electron transfer is reported to be hindered by Tween 80 (Lu & Levin, 1974). There is also some hydroxylase activity with cytochrome *P*-450 alone. This effect can be explained by the contamination of the cytochrome *P*-450 preparation with traces of NADPH:cytochrome *c* (*P*-450) reductase. But, nevertheless, we were able to reconstitute the digitoxin 12 β -hydroxylase *in vitro* from cytochrome *P*-450, NADPH:cytochrome *c* (*P*-450) reductase and the naturally occurring microsomal lipids. The assay has to be carried out in the presence of FAD and FMN.

DISCUSSION

In order to provide data on the molecular constituents interacting during a cytochrome *P*-450-dependent hydroxylation reaction in plant cells, it is absolutely essential to solubilize and purify the enzyme proteins before any attempt to reconstitute the whole complex.

One grave problem during solubilization and purification is the instability of cytochrome *P*-450, which is converted into its inactive form, cytochrome *P*-420. The best results were achieved with CHAPS and with the addition of 15% (v/v) glycerol. At detergent concentrations of about 1%, both major constituents of the digitoxin 12 β -hydroxylase, cytochrome *P*-450 and NADPH:cytochrome *c* (*P*-450) reductase seemed to be localized to different micella; this facilitated the purification of cytochrome *P*-450 as well as of NADPH:cytochrome *c* (*P*-450) reductase. Fortunately the separation of both proteins by ion-exchange chromatography was very good, since cytochrome *P*-450 was not bound to DEAE-Sephacel and was eluted by KCl-free buffer, whereas the activity of NADPH:cytochrome *c* (*P*-450) reductase was eluted at a KCl concentration of 0.2 M. The purification of cytochrome *P*-450 was only

6-fold. Such low purification was also observed for other similar plant-derived systems. Gabriac *et al.* (1985) reported a 6.9-fold, and Higashi *et al.* (1985) a 6-fold, purification of cytochrome *P*-450. The low yield of about 30% might be due to the inactivation of cytochrome *P*-450. There was no indication of the presence of cytochrome *b*₅ in the peak fractions of cytochrome *P*-450.

NADPH:cytochrome *c* (*P*-450) reductase was further purified by affinity chromatography. This purification step led to a 248-fold purification of the reductase, which was the only protein visible after SDS/PAGE and silver staining. Since the reductase is a flavoprotein, the flavin groups, which are lost during the purification procedure, could be replaced by exogenous FAD and FMN, which resulted in a 9.7-fold stimulation of the enzyme activity. Such a stimulation is also reported for NADPH:cytochrome *c* (*P*-450) reductase from other plant systems (Benveniste *et al.*, 1986; Madyastha & Coscia, 1979). Further stimulation could be achieved by addition of 0.1% of the non-ionic detergent Tween 80 during the purification procedure or to the test assay. Obviously the zwitterionic detergent CHAPS does not offer an appropriately lipophilic environment to the reductase, but this is fulfilled by Tween 80. Unfortunately this detergent is reported to interfere with the substrate binding and electron transport to the cytochrome *P*-450 (Lu & Levin, 1974) and is therefore unsuitable for reconstitution experiments.

For reconstitution experiments, microsomal lipids from *Digitalis lanata* were used in order to provide a largely natural environment for the constituents of the enzyme.

The lipids were extracted from *Digitalis lanata* microsomes with chloroform/methanol. The major components of the membrane matrix were phosphatidylethanolamine and phosphatidylcholine. The microsomal lipids were sonicated in buffer to form liposomes and were applied as matrix material for integration of the partially purified constituents of the digitoxin 12 β -hydroxylase. For reconstitution the partially purified cytochrome *P*-450 and the homogeneously purified NADPH:cytochrome *c* (*P*-450) reductase were added to the liposomes in presence of the flavin nucleotides FAD and FMN. After this reconstitution, 10% of the initial microsomal digitoxin 12 β -hydroxylase activity was detectable. Similar results were reported for the monoterpene hydroxylase in *Catharanthus roseus* (Madyastha *et al.*, 1976). In the case of cinnamic acid 4-hydroxylase from *Helianthus tuberosus* (Benveniste *et al.*, 1986), only 0.3% of the initial activity was achieved. In all these experiments substantial damage to the protein molecules has to be assumed. One also has to bear in mind that the applied cytochrome *P*-450 is not specific for digitoxin. Probably only a minor part of the total microsomal cytochrome *P*-450 of *Digitalis lanata* is specific for the hydroxylation of digitoxin; other isoenzymes are, for example, specific for cinnamic acid hydroxylation. Therefore the catalytic-centre activity in the reconstituted assay is not very high. Another problem is the degree of integration of the purified proteins into the liposomes. We did not determine the amount of free cytochrome *P*-450 or free reductase, but this amount might be rather high. These proteins not integrated in liposomes do not contribute to the hydroxylation reaction.

Another explanation of the low hydroxylation activity

could be the removal of other redox systems during purification and reconstitution. The participation of cytochrome *b*₅ and the corresponding reductase has been discussed for animal systems, but is still very doubtful (Blanck *et al.*, 1984). It was proved that cytochrome *b*₅ was not present in the reconstitution assays. In experiments with whole microsomes, the participation of cytochrome *b*₅ and a NADH-dependent reductase was shown to be unlikely, since no synergistic effect appeared with NADH and NADP⁺ or NADPH. Up to now a participation of cytochrome *b*₅-dependent systems in plant microsomal *P*-450-dependent reactions has not been demonstrated (West, 1980).

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