Characterization and Localization of Digitoxin 12β-Hydroxylase from Cell Cultures of *Digitalis lanata* EHRH

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The cytochrome P-450-dependent monooxygenase digitoxin 12β-hydroxylase from cell cultures of *Digitalis lanata* needs NADPH and molecular oxygen and hydroxylates cardiac glycosides with the aglycon of digitoxigenin to the corresponding derivatives of the C-series. Other electron donors cannot replace NADPH. The apparent K_m-values are 26 μM for NADPH, 7.1 μM for β-methyldigitoxin and 10 μM for digitoxin. The reaction is inhibited by $NADP^+$ and cytochrome *c* in a competitive mode. The optimum temperature was at 20 °C. Low concentrations of Mn^{2+}, Mg^{2+}, and EDTA were slightly stimulatory, but there was no strict dependence on divalent cations. Digitoxin 12β-hydroxylase is very stable at room temperature and the reaction proceeds for more than 20 h. After the addition of 15% glycerol, 70% of the original activity can be retained subsequent to freezing at −18 °C. By means of linear sucrose gradient fractionation of cellular membranes the digitoxin 12β-hydroxylase was found to be located in the endoplasmic reticulum.

Introduction

Since the first cytochrome P-450-dependent enzyme in plants which catalyses hydroxylation steps during gibberellin biosynthesis was detected in 1969 by Murphy and West [1] our knowledge about cytochrome P-450-dependent plant enzymes has continued to increase. Most of these enzymes in plant cells catalyse biosynthetic steps leading to secondary products or growth regulators. The best-characterized enzyme is cinnamic acid 4-hydroxylase [2–5], others are ferulic acid 5-hydroxylase [6], and flavonoid 3′-hydroxylase [7, 8], which are part of the biosynthetic pathways to phenylpropanoids, lignins, flavonoids and anthocyanins. Monoterpene hydroxylase from *Catharanthus roseus* [9, 10], which is involved in alkaloid biosynthesis, and ipomeamarone 15-hydroxylase [11] and pterocarpan 6a-hydroxylase [12], which is necessary for phytoalexin biosynthesis are also cytochrome P-450-dependent. Cytochrome P-450-dependent enzymes can also catalyse the biosynthesis or modification of growth regulators. Several steps in gibberellin biosynthesis are dependent on cytochrome P-450 [1, 13, 14]; the hydroxylation of abscisic acid to phaseic acid [15] and the hydroxylation of cytokinins [16], as well as modifications of 2.4-D [17] are known or thought to be dependent on cytochrome P-450. Most of these enzymes are located in the endoplasmic reticulum; an exception is the monoterpene hydroxylase from *Catharanthus roseus* which was detected in a provacuolar fraction [18].

As previously described [19] digitoxin 12β-hydroxylase from cell cultures of *Digitalis lanata* is a membrane-bound and cytochrome P-450-dependent enzyme [19].

Materials and Methods

Cell suspension cultures

Cell cultures of *Digitalis lanata* were cultivated as described elsewhere [19].

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Chemicals

β-Methyldigoxin was purchased from Roth (Karlsruhe, FRG), and the digitoxin and digoxin were from Serva (Heidelberg, FRG). β-Methyldigitoxin and all the other cardiac glycosides were from our laboratory collection. $NADP^+$ was obtained from Fluka (Neu-Ulm, FRG), the NADPH was from Sigma (Munich, FRG) and the glucose-6-phosphate as well as glucose-6-phosphate dehydrogenase from Boehringer (Mannheim, FRG).

Preparation of microsomes

The microsomes were prepared as previously described [19] with the only exception that instead of phosphate buffer a Tris·HCl buffer was used (0.1 M Tris·HCl pH 7.5 with 1 mM EDTA, 1 mM DTT, and 0.6 M mannitol). The cells were harvested 4 to 6 days after onset of cultivation and ground in a precooled mortar with 1 ml of the buffer together with quartz sand.

Sucrose density gradients

Crude homogenates for sucrose density gradient centrifugation were obtained by grinding the cells in 0.3 ml buffer (0.1 M Tris·HCl pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% sucrose) per g fresh weight. The supernatant (5 ml) of a $8000 \times g$ centrifugation was layered on 33.5 ml of a linear gradient from 15 to 45% sucrose. The gradients were centrifuged for 3 h in a SW 27 rotor at 25000 rpm. They were fractionated into 2.2 ml fractions and assayed for digitoxin 12β-hydroxylase activity and for marker enzymes.

Digitoxin 12β-hydroxylase activity

The digitoxin 12β-hydroxylase was assayed as already described [19] with some minor variations: the protein amount in the assay was 2.5 mg and the NADP-regenerating system consisted of 1 mM $NADP^+$, 5 mM glucose-6-phosphate and 8.4 nkatal·ml^{-1} glucose-6-phosphate dehydrogenase. When the gradient fractions were assayed the volume was reduced to 1 ml and the extraction procedure was performed with 3 ml chloroform.

Determination of marker enzymes

Glucansynthase I with the substrates UDP-glucose or GDP-glucose were measured according to [20], as was glucansynthase II. Latent IDPase was determined according to [21] and KCl-stimulated ATPase activity according to [22]. Cytochrome *c* oxidase and NADH-cytochrome *c* reductase were assayed according to [23].

Other measurements

Sucrose concentrations were measured with a refractometer (Zeiss, Oberkochen, FRG). Protein concentrations were determined according to [24] with bovine serum albumin as the standard. The cytochrome P-450 content of microsomes was measured according to [25], the cytochrome b_5 content according to [26].

Results

Cytochrome P-450

In microsomal preparations cytochrome P-450 could be detected by examining the CO-difference spectra of sodium dithionite-reduced microsomes. The CO-difference spectra showed two prominent peaks at 420 and 450 nm (Fig. 1), which demonstrate

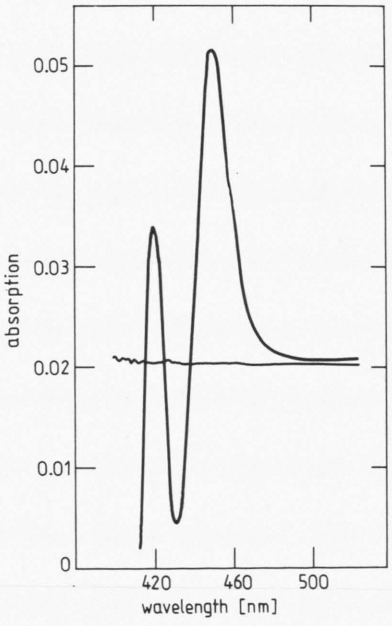

Fig. 1. CO-difference spectrum of sodium dithionite-reduced microsomes. The reduced state of the membranes is represented by the baseline. The sample was treated with CO and the difference spectrum was recorded. The peak at 450 nm was used to calculate the cytochrome P-450 content of the membranes.

the occurrence of cytochrome P-420 and P-450. Cytochrome P-420, which is the inactive form of cytochrome P-450 is thought to be a denatured product formed during the preparation of the microsomes but also other heme-ligated compounds could contribute to this peak. The cytochrome P-450 content of the microsomes could be calculated with the help of these difference spectra according to [25]. It varied between 100 and 250 pmol per mg of microsomal protein but tended to be between 110 and 140 pmol · mg^{-1}. If we compare these values with the cytochrome P-450 contents of various plants published by Rich and Bendall [27], we see that *Digitalis lanata* microsomes contain a relatively large amount of cytochrome P-450. Reports on cytochrome P-450-dependent enzymes show values of about 100 pmol and more [3, 4, 8, 10, 12, 14].

The difference spectra of oxidized and NADH-reduced *Digitalis lanata* microsomes showed a relatively high cytochrome b_5 content. It ranged from 120 to 160 pmol per mg microsomal protein. These levels are very similar to those found in aging *Helianthus tuberosus* tissue [28, 29].

Temperature and pH optima

As already reported the pH optimum for digitoxin 12β-hydroxylase was found to be pH 7.5. This was verified using different buffer systems, Tris · HCl and KH_2PO_4/K_2HPO_4. This slightly alkaline pH optimum corresponds with most data for other cytochrome P-450-dependent enzymes [2, 4, 6, 9, 12, 30]. The optimum temperature for digitoxin 12β-hydroxylase is 20 °C. At higher temperatures the reaction declines, possibly due to the higher membrane fluidity which makes the co-operation between NADPH-cytochrome *c* (P-450) reductase and cytochrome P-450 more difficult.

Reaction kinetics and stability

As shown in Fig. 2 the reaction catalysed by digitoxin 12β-hydroxylase proceeds linearly up to an incubation time of 4 h. The hydroxylation of β-methyldigitoxin proceeds strongly up to a reaction time of 20 h, the longest period tested, but the reaction had not yet stopped after this incubation period. This demonstrates the high stability of the digitoxin 12β-hydroxylase in contrast to the lability of most of the other cytochrome P-450-dependent enzymes [12, 30]. Nevertheless, digitoxin 12β-hydroxylase is very

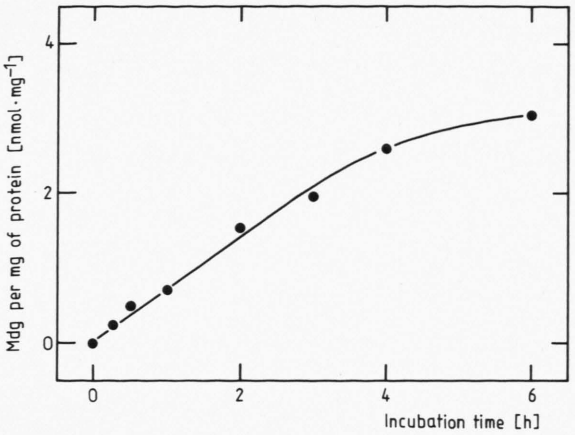

Fig. 2. Enzyme kinetics of the digitoxin 12β-hydroxylase. The enzyme was assayed by the standard procedure. The resulting β-methyldigoxin was measured on the basis of microsomal protein.

sensitive to freezing, which reduced the activity by more than 70%. The addition of 15% glycerol stabilized the enzyme, leading to 70% recovery of the initial activity after storage for 8 days at −18 °C. Nearly the total enzyme activity was retained after storage for 24 h on ice.

With freely suspended microsomes the hydroxylation of β-methyldigitoxin to β-methyldigoxin proceeds linearly up to a protein concentration of 0.75 mg protein per ml assay volume.

Co-factor requirements

The co-factor requirements of the digitoxin 12β-hydroxylase have been shown in a preliminary publication [19]. The enzyme is dependent on NADPH and is saturated by a concentration of 1 mM NADPH supplied directly or *via* a regenerating system. From a Hanes plot (Fig. 3) an apparent K_m value for NADPH of 26 μM was calculated. A typical substrate saturation curve is obtained for NADPH with a slight substrate inhibition at concentrations exceeding 1 mM. A K_m value of 15.2 μM was determined for NADPH-cytochrome *c* (P-450) reductase from *Digitalis lanata* microsomes, which was purified to homogeneity by ion exchange and affinity chromatography [31]. Therefore the K_m values for NADPH and NADPH-cytochrome *c* (P-450) reductase, a putative constituent of the hydroxylase, are very similar.

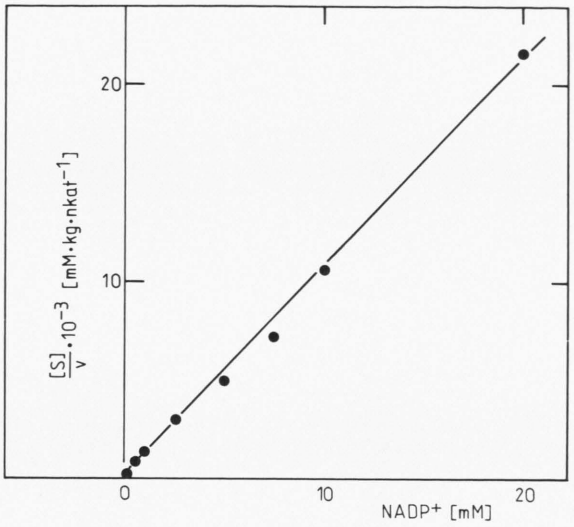

Fig. 3. Hanes plot for digitoxin 12β-hydroxylase at increasing concentrations of NADPH. NADPH was applied *via* a regenerating system consisting of 5 mM glucose-6-phosphate, 8.4 nkatal·ml^{-1} glucose-6-phosphate dehydrogenase, and the indicated $NADP^+$ concentrations. The K_m value for NADPH was calculated to be 26 μM.

Inhibition by $NADP^+$

Digitoxin 12β-hydroxylase, which needs NADPH for hydroxylation, is competitively inhibited by $NADP^+$. Fig. 4 shows the Hanes plot of a set of experiments with varying NADPH and $NADP^+$ concentrations. It shows the competitive mode of inhibition. $NADP^+$ seems to compete with NADPH for the active site at the NADPH-cytochrome *c* (P-450) reductase.

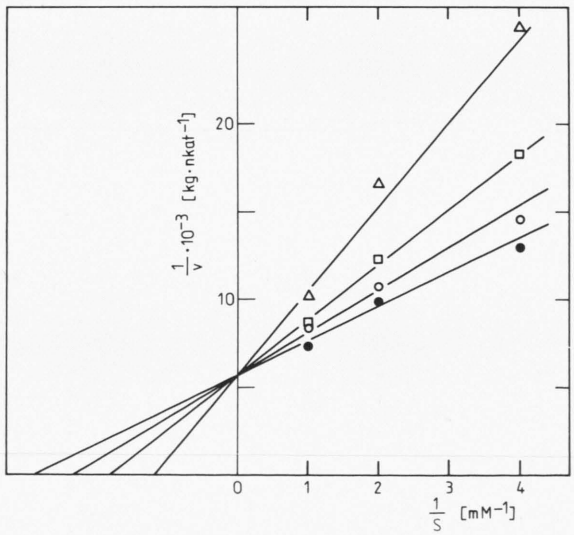

Fig. 4. Competitive inhibition of digitoxin 12β-hydroxylase by $NADP^+$ (Hanes plot). A K_i value of 1.1 mM was calculated. ●—● without $NADP^+$, ○—○ 0.5 mM $NADP^+$, □—□ 1 mM $NADP^+$, and △—△ 2 mM $NADP^+$.

Substrate specificity

Standard incubations for digitoxin 12β-hydroxylase were performed with β-methyldigitoxin, though this compound is not the natural substrate. In biotransformation experiments with *Digitalis lanata* cell cultures, β-methyldigitoxin is used as a substrate, because the chemically introduced methyl group inhibits the glucosylation also performed by cell cultures *in vivo* and strongly promotes the desired hydroxylation reaction. In order to facilitate the comparison of *in vivo* and *in vitro* data β-methyldigitoxin is used in the assay. Digitoxin 12β-hydroxylase is saturated at a concentration of 20 μM β-methyldigitoxin; a K_m value of 7.1 μM can be calculated [19]. For digitoxin, the supposed natural substrate, the K_m value is 10 μM. A very slight substrate inhibition can be observed at higher concentrations of cardiac glycosides. Inhibition by the reaction product β-methyldigoxin or digoxin was never observed even when the product concentration was fivefold of the substrate concentration.

β-Methyldigitoxin and digitoxin are not the only cardiac glycosides which can be hydroxylated by the digitoxin 12β-hydroxylase. All of the substrates tested which have the same aglycon as digitoxin were hydroxylated, irrespective of the number and kind of sugars at the C_3 of the aglycon. Digitoxin 12β-hydroxylase hydroxylated digitoxigenin, digitoxigenin monodigitoxosid, digitoxigenin bisdigitoxosid and acetyldigitoxin to their corresponding derivatives of the C-series. No hydroxylation could be observed, on the other hand, for gitoxigenin, K-strophantin-β and cymarin, all cardiac glycosides with a different aglycon. This shows that the substrate specificity is based on the recognition of the aglycon.

Inhibition by cytochrome c

A typical characteristic of cytochrome P-450-dependent enzymes is the inhibition by cytochrome *c*, since one constituent of the enzyme system, NADPH-cytochrome *c* (P-450) reductase, can transfer electrons to cytochrome *c*. NADPH-cytochrome *c* (P-450) reductase is commonly used as a marker

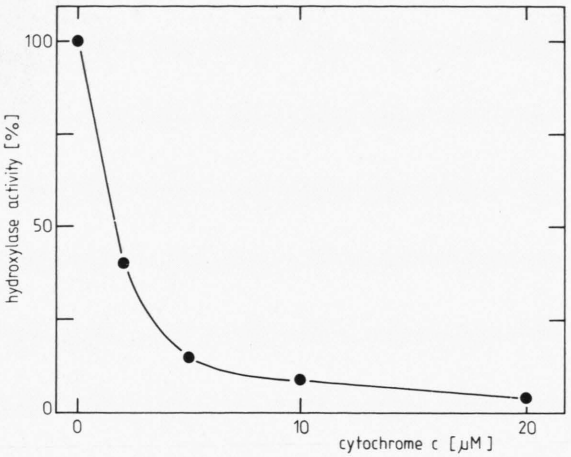

Fig. 5. Inhibition of digitoxin 12β-hydroxylase by cytochrome *c*. Cytochrome *c* was added to the standard assay as indicated.

enzyme for the endoplasmic reticulum with cytochrome *c* as a substrate. Fig. 5 shows the inhibition of digitoxin 12β-hydroxylase activity by cytochrome *c*. Total inhibition is achieved at a concentration of 20 μM cytochrome *c*. The mode of inhibition is thought to be a competitive one, since cytochrome *c* competes for the electrons transferred from NADPH *via* the reductase.

Effects of divalent cations

The effects of several divalent cations as well as EDTA on digitoxin 12β-hydroxylase activity were tested. The ions were applied at concentrations of 0.2 and 2 mM. $CoCl_2$, $ZnCl_2$, and $HgCl_2$ were strongly inhibitory. $CaCl_2$ inhibited the enzyme activity by about 40% at a concentration of 2 mM. $MnCl_2$ stimulated the enzyme activity by 40% at a concentration of 0.2 mM. Low concentrations of $MgCl_2$ had also a stimulatory effect, but concentrations exceeding 1 mM caused increasing inhibition. Thus it would seem to be contradictory to the fact that 1 mM EDTA should also stimulate digitoxin 12β-hydroxylase activity [19]. An explanation can be found in the fact that microsomes were prepared by $MgCl_2$-precipitation. The very high concentration of $MgCl_2$, 50 mM, strongly inhibits the enzyme. This inhibition is prevented by the addition of 1 mM EDTA, but any higher EDTA concentration begins to inhibit digitoxin 12β-hydroxylase activity itself.

Localization of digitoxin 12β-hydroxylase

By means of differential centrifugation experiments it was shown that digitoxin 12β-hydroxylase activity was present in a microsomal fraction [19]. In order to get more detailed information on the localization of the enzyme, crude homogenates of *Digitalis lanata* cells were centrifuged on linear sucrose gradients. After the fractionation of the gradients the activities of several marker enzymes and of digitoxin 12β-hydroxylase were determined in the separate fractions. As shown in Fig. 6, the distribution of NADH cytochrome *c* reductase, a marker enzyme for the endoplasmic reticulum, corresponds perfectly

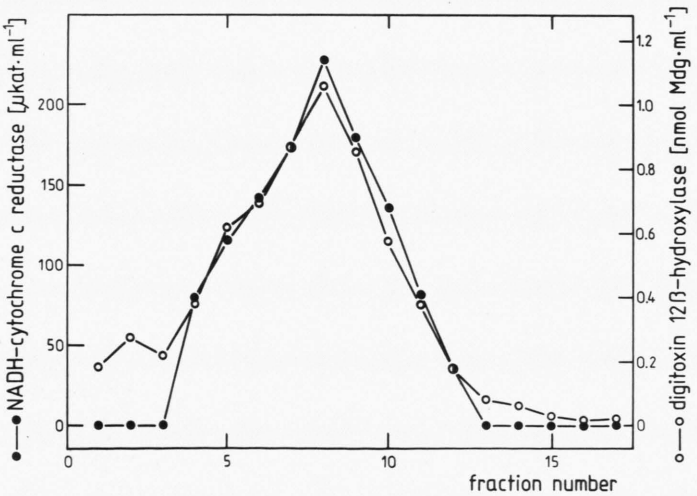

Fig. 6. Sucrose density gradient fractionation of digitoxin 12β-hydroxylase compared to the distribution of NADH-cytochrome *c* reductase activity as an ER marker enzyme. Sucrose gradient: 15–45% (w/w). Centrifugation conditions: 25000 rpm in a Beckman SW 27 rotor. 5 ml of a 8000 × *g* supernatant from crude cell preparations were layered on the gradient. ●—● digitoxin 12β-hydroxylase activity, ○—○ NADH cytochrome *c* reductase.

with the distribution of digitoxin 12β-hydroxylase in the gradient. The maximum activity for both enzymes was found at a sucrose concentration of 27%, which means a density of 1.11 $g \cdot cm^{-3}$. The marker enzymes for mitochondria, cytochrome *c* oxidase, for the Golgi apparatus, latent IDPase and glucan synthase I, and for plasma membrane, glucan synthase II, show a different distribution and other maxima (see Fig. 7 and 8). Thus, digitoxin 12β-hydroxylase seems to be located in the endoplasmic reticulum, a localization also found for most other cytochrome P-450-dependent enzymes from higher plants [3, 32, 33, 34].

Discussion

This publication deals with the characterization and localization of a new cytochrome P-450-dependent monooxygenase from higher plants, digitoxin 12β-hydroxylase. It is the first enzyme found in higher plants that catalyses the hydroxylation of steroid-like molecules, which is a typical cytochrome P-450-catalyzed reaction in the liver of mammals. It is interesting to note that the same reaction mechanism is used by the higher plant *Digitalis lanata*. The typical characteristics of the cytochrome P-450-catalysed reaction coincide with those of mammalian enzymes of this type [19], the inhibition by CO and the rever-

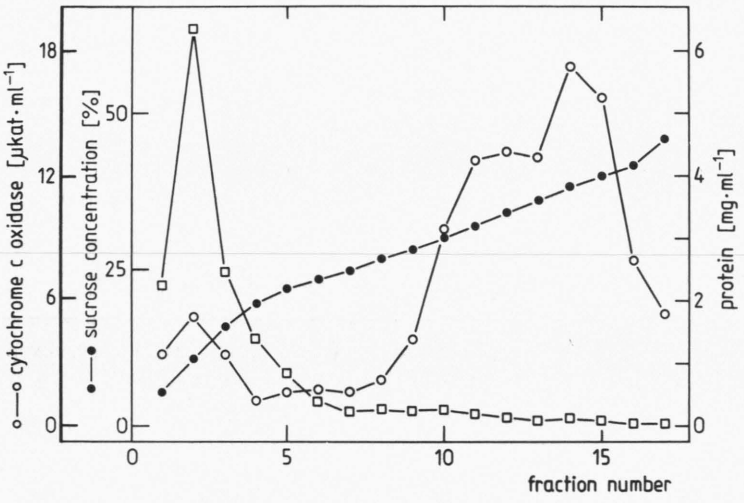

Fig. 7. Distribution pattern of protein and cytochrome *c* oxidase in a linear sucrose gradient ranging from 15 to 45% (w/w). For centrifugation conditions see Fig. 6. ●—● sucrose gradient, □—□ protein, ○—○ cytochrome *c* oxidase activity.

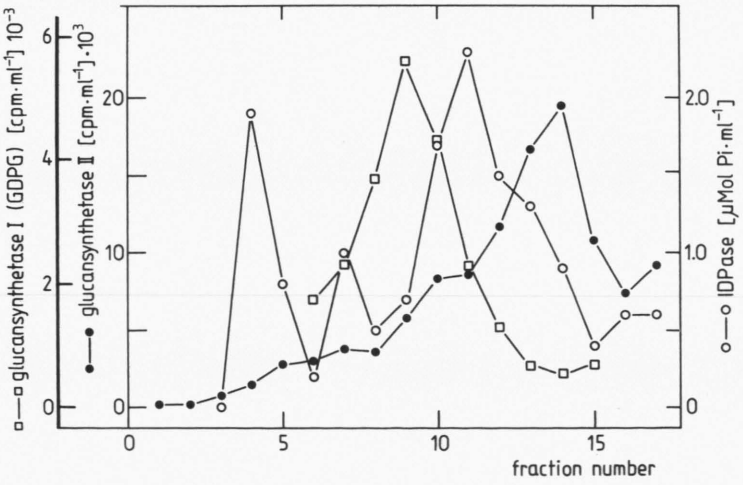

Fig. 8. Distribution of marker enzymes in a linear sucrose gradient (15–45%; w/w). Glucan synthase I □—□, glucan synthase II ●—●, latent IDPase ○—○. For centrifugation conditions see Fig. 6.

sion of the inhibition by illumination with 450 nm light. The general characteristics of the digitoxin 12β-hydroxylase such as the slightly alkaline pH optimum, the substrate dependencies, inhibition by $NADP^+$ and cytochrome *c*, inhibition by Hg^{2+}, Cu^{2+}, and *p*-hydroxymercuribenzoate and the insensitivity to KCN are very similar to most other properties of cytochrome P-450-dependent enzymes from other plants. The missing synergistic effect of NADPH or $NADP^+$ and NADH indicates the absence of transhydrogenases in the enzyme preparation and the status of NADPH as the only electron donor [19]. A cytochrome b_5-dependent system transferring electrons from NADH to cytochrome P-450 is therefore improbable although a substantial amount of cytochrome b_5 could be measured in the microsomal membranes. The participation of cytochrome b_5-dependent electron transfer in cytochrome P-450-dependent reactions has been shown in mammalian liver systems but is still difficult to establish in higher plant systems. A missing synergistic effect has also been reported for the lauric acid α-hydroxylase from pea seedlings [35] and *Helianthus tuberosus* [36]. One peculiarity of the digitoxin 12β-hydroxylase is its high stability at room temperature and the fact that the hydroxylation reaction proceeds for more than 20 h. Most other cytochrome P-450-dependent plant enzymes are reported to be very unstable at room temperature and loose their activities within short periods [12, 30]. This stability of the digitoxin 12β-hydroxylase is a prerequisite for the application of the isolated enzyme in transforming cardiac glycosides of the A-series to the C-series. For that purpose an immobilized enzyme system with the digitoxin 12β-hydroxylase enclosed in a Ca-alginate matrix was described [37].

As is true for most other cytochrome P-450-dependent enzymes from higher plants the digitoxin 12β-hydroxylase is located in the endoplasmic reticulum. This could be demonstrated by the sucrose density fractionation of cellular membranes. This distribution of digitoxin 12β-hydroxylase and a marker enzyme for the endoplasmic reticulum, NADH cytochrome *c* reductase, was identical. So far, only one cytochrome P-450-dependent plant enzyme has been reported not to be located in the endoplasmic reticulum but in provacuolar membranes, the monoterpene hydroxylase from *Catharanthus roseus* [18].

We now tend to solubilize, purify and reconstitute the digitoxin 12β-hydroxylase.

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