CHARACTERIZATION OF CYTOCHROME $P450_{TYR}$, A MULTIFUNCTIONAL HAEM-THIOLATE *N*-HYDROXYLASE INVOLVED IN THE BIOSYNTHESIS OF THE CYANOGENIC GLUCOSIDE DHURRIN

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SUMMARY

The haem-thiolate *N*-hydroxylase cytochrome $P450_{TYR}$ involved in the biosynthesis of the tyrosine-derived cyanogenic glucoside dhurrin in *Sorghum bicolor* had recently been isolated. Reconstitution of enzyme activity by insertion of cytochrome $P450_{TYR}$ and NADPH-cytochrome P450-reductase into L-α-dilauroylphosphatidylcholine micelles and using tyrosine as substrate results in the formation of *p*-hydroxyphenylacetaldehyde oxime. Quantitative substrate binding spectra demonstrate that tyrosine and *N*-hydroxytyrosine are mutually exclusive substrates that bind to the same active site of cytochrome $P450_{TYR}$. The multifunctionality of cytochrome $P450_{TYR}$ has been confirmed in reconstitution experiments using recombinant cytochrome $P450_{TYR}$ expressed in *Escherichia coli*. It was earlier reported that an *in vitro* microsomal system catalyzing all but the last step in the biosynthetic pathway for cyanogenic glucosides exhibits catalytic facilitation (channelling). This observation is explained by the multifunctionality of cytochrome $P450_{TYR}$. The cytochrome $P450_{TYR}$ sequence represents the first amino acid sequence of a functionally characterized cytochrome P-450 enzyme from a monocotyledonous plant and the first sequence of an N-hydroxylase with high substrate specificity. Multifunctional N-hydroxylases of the cytochrome P-450 type have not previously been reported in living organisms.

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KEY WORDS

cyanogenic glucosides, multifunctional, N-hydroxylation, cytochrome P450

INTRODUCTION

The ability of living organisms to produce hydrogen cyanide (HCN) is known as cyanogenesis. This phenomenon is exhibited by numerous higher plants and microorganisms (fungi and bacteria) and by some animals (myriapods and insects) (for review see /1/). The source of release of HCN is most often cyanogenic glucosides, but a few examples of cyanogenic lipids have been reported /1/. Cyanogenic glucosides are found in approximately 2500 plant species, including the agriculturally important crop plants sorghum (*Sorghum bicolor* L. Moench) and cassava (*Manihot esculenta* Crantz). Insufficient removal of the cyanogenic glucosides present in cassava roots constitutes a potential health hazard for the millions of people who are dependent on these roots as their major staple food /2,3/. Although not documented cyanogenic glucosides are thought to play a role in non-specific plant defence mechanisms. However, in some cyanogenic plants the HCN released is more harmful to the host plant than to the pest organism. This is the case in the cyanogenic rubber tree (*Hevea brasiliensis*), in which HCN release is shown to inhibit production of the phytoalexin scopoletin resulting in an increased susceptibility to attack by the fungus *Microcyclus ulei* /4/. Similarly, the presence of the cyanogenic glucoside *epi*-heterodendrin in the epidermal cells of barley (*Hordeum vulgare*) seedlings is correlated with an increased sensitivity to the mildew fungus *Erysiphe graminis* /5/. The presence of *epi*-heterodendrin in barley malt causes a problem in the brewing industry, because cyanide and ethanol can form the carcinogenic ethylcarbamate during the distilling process /6/. The possibility of regulating and optimizing the level of cyanogenic glucosides in specific plant tissues could potentially provide increased disease and pest resistance as well as improved nutritional value of foods.

We have used sorghum as a model plant to study the biosynthesis of cyanogenic glucosides. We report our results towards elucidation of the biosynthetic pathway and towards the characterization of the enzymes and the structural genes involved.

THE BIOSYNTHETIC PATHWAY

Cyanogenic glucosides are derived from five hydrophobic protein amino acids, and a single non-protein amino acid /7/. Sorghum contains the cyanogenic glucoside dhurrin derived from tyrosine. A biosynthetically active microsomal enzyme system has been isolated from etiolated seedlings of sorghum. The microsomal enzyme system catalyzes the conversion of L-tyrosine to *p*-hydroxymandelonitrile /8/. *In vivo*, *p*-hydroxymandelonitrile is glycosylated by a soluble UDPG-glucosyltransferase to dhurrin, which accumulates in the vacuole. When the microsomal enzyme system is prepared in the absence of thiol reagents (-SH microsomal preparations), the last part of the pathway is inactivated and tyrosine is converted only to the oxime /9/. Using the +SH and -SH microsomal enzyme systems it has earlier been demonstrated that the key intermediates in the biosynthetic pathway are *N*-hydroxytyrosine, (*E*)- and (*Z*)-*p*-hydroxyphenylacetaldehyde oxime, *p*-hydroxyphenylacetonitrile, and *p*-hydroxymandelonitrile (Fig. 1) /9-11/. All the intermediates have been shown to be produced and metabolized by the enzyme system. Stoichiometric measurements of oxygen consumption and biosynthetic activity have demonstrated that the pathway involves three hydroxylation reactions /12/. Two molecules of oxygen are consumed in the conversion of tyrosine to *p*-hydroxyphenylacetaldehyde oxime and one oxygen molecule is consumed in the conversion of *p*-hydroxyphenylacetonitrile to *p*-hydroxymandelonitrile /12/. The N-hydroxylation of tyrosine and the C-hydroxylation of *p*-hydroxyphenylacetonitrile have been shown to be catalyzed by cytochrome P450-dependent monooxygenases /13/. Based on the similarity of the biosynthetic reactions in different cyanogenic plants, it is believed that the information obtained with the sorghum system can be extended to other cyanogenic plants.

PURIFICATION OF CYTOCHROME $P450_{TYR}$

For identification of the enzymes catalyzing the biosynthesis of cyanogenic glucosides, we have taken the classical biochemical approach to purify the proteins. Generally, the isolation of plant cytochromes P450 has proved difficult because the proteins are only present in minute amounts and only at specific developmental stages and/or environmental conditions. While total cytochromes P450 constitute approximately 10% of the protein in rat liver microsomes,

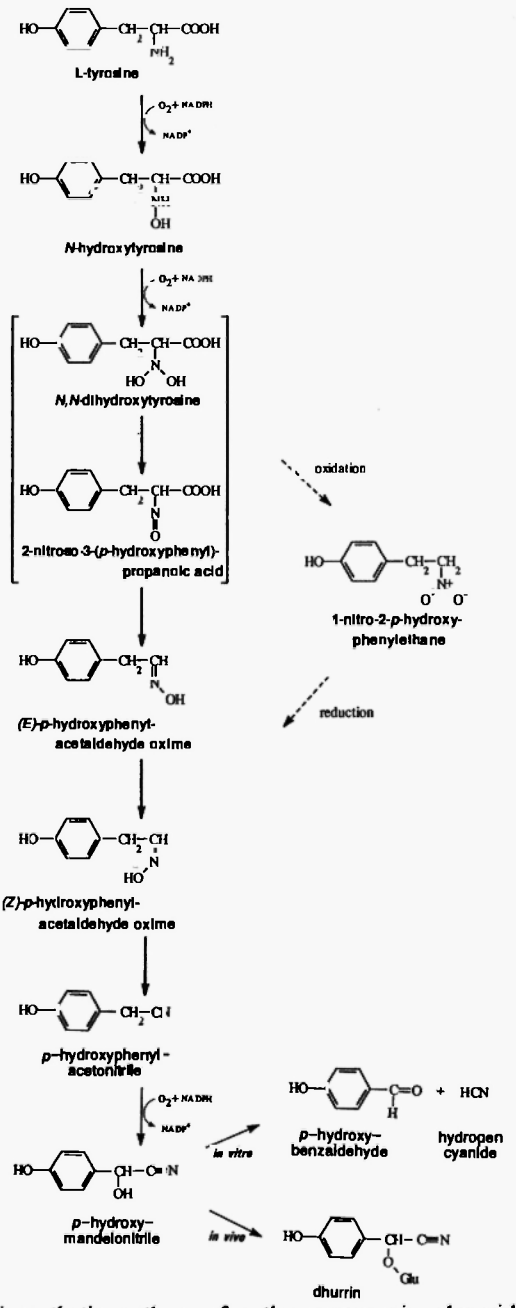

Fig. 1: The biosynthetic pathway for the cyanogenic glucoside dhurrin in *Sorghum bicolor*. Cytochrome P450$_{TYR}$ catalyzes the conversion of tyrosine to *p*-hydroxyphenylacetaldehyde oxime.

total cytochromes P450 in microsomes isolated from sorghum seedlings constitute less than 1% /14/. For the isolation of cytochrome $P450_{TYR}$, the first enzyme in the dhurrin pathway, a new procedure was developed based on dye affinity chromatography (Fig. 2) /14/. Microsomal preparations from etiolated sorghum seedlings were used as starting material. The isolation procedure was designed to avoid high concentrations of the cytochrome P450 enzymes to prevent irreversible aggregation. The first step in the purification procedure was solubilization of the microsomal membranes using a mixture of the non-ionic detergents RENEX 690 and RTX-100 followed by ultracentrifugation at 200,000 *g*. The supernatant containing the solubilized enzymes was applied to a column packed with DEAE Sepharose fast flow/S-100 Sepharose (20:80). Yellow pigments were removed by an extensive wash in RENEX 690-containing buffer. The total cytochromes P450 were eluted by increasing the EDTA

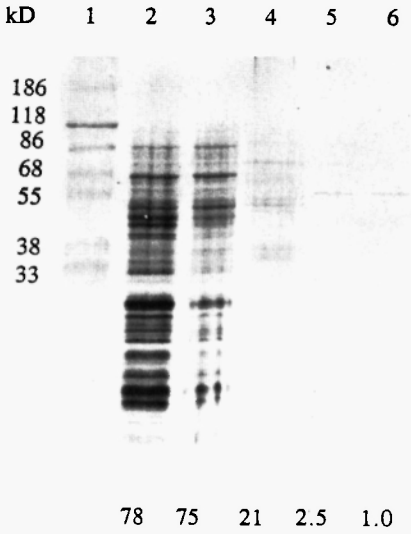

Fig. 2: The polypeptide composition of the fractions obtained during isolation of cytochrome $P450_{TYR}$ as monitored by SDS-PAGE and staining with Coomassie. Identical amounts of total cytochrome P450 (7 pmol) were applied per lane. The total yields of cytochromes P450 in a typical experiment are indicated.

concentration from 0.2 mM to 5 mM with a buffer containing RENEX 690 and CHAPS. Under these conditions the NADPH-cytochrome P450 reductase and cytochrome b_5 remained bound to the column and were eluted separately using a KCl-gradient. A homogeneous reductase was obtained by affinity chromatography on 2′,5′-ADP-Sepharose. Cytochrome b_5 was purified to homogeneity by hydroxyapatite chromatography.

The total cytochromes P450 eluted from the ion exchange column were applied to a column containing Reactive Yellow 3. Under the experimental conditions used, cytochrome P450$_{TYR}$ did not bind to the Reactive Yellow 3 matrix, whereas a major part of the ubiquitous cinnamic acid 4-hydroxylase was bound. The run-off was collected and applied to Cibachron Blue Agarose from which cytochrome P450$_{TYR}$ was eluted using a phosphate gradient. As a final step cytochrome P450$_{TYR}$ was applied to a column containing Reactive Red 120 Agarose. A highly purified cytochrome P450$_{TYR}$ was eluted from the column with a KCl gradient (Fig. 2). During fractionation the total cytochrome P450 content of the individual fractions was quantified by carbon monoxide difference spectrometry /15/. Cytochrome P450$_{TYR}$ was monitored by its specific ability to form type I substrate binding spectra using tyrosine as substrate (Fig. 3) /12/. The molecular mass of cytochrome P450$_{TYR}$ is 57 kDa as determined by SDS-PAGE (Fig. 2). For production of polyclonal antibodies and determination of amino acid sequences, cytochrome P450$_{TYR}$ was further purified by preparative SDS-PAGE.

CHARACTERIZATION OF CYTOCHROME P450$_{TYR}$

Reconstitution of the cytochrome P450$_{TYR}$ monooxygenase was carried out by insertion of purified cytochrome P450$_{TYR}$ and NADPH-cytochrome P450 reductase into artificial lipid vesicles consisting of L-α-dilauroylphosphatidylcholine. Addition of tyrosine and NADPH to the reconstituted monooxygenase results in the production of *p*-hydroxyphenylacetaldehyde oxime /17/. This indicates that cytochrome P450$_{TYR}$ catalyzes both N-hydroxylation steps between tyrosine and the oxime. In accordance with this result, cytochrome P450$_{TYR}$ is able to bind *N*-hydroxytyrosine forming a type I binding spectrum. Quantification of substrate binding spectra using both tyrosine and *N*-hydroxytyrosine as substrates shows that sequential addition of *N*-hydroxytyrosine to cytochrome P450$_{TYR}$ saturated with tyrosine or *vice*

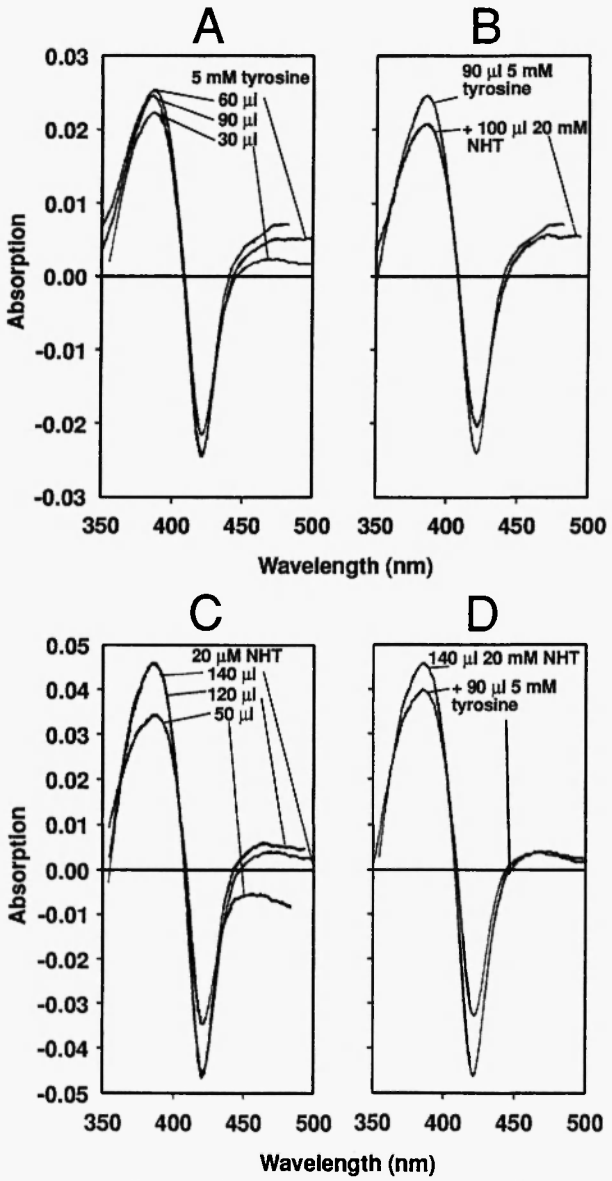

Fig. 3: Substrate binding spectra of cytochrome $P450_{TYR}$ using tyrosine and *N*-hydroxytyrosine as substrates. 5 mM tyrosine or 20 mm *N*-hydroxytyrosine were added as indicated. A: Titration with tyrosine; B: saturation with tyrosine; then titration with *N*-hydroxytyrosine; C: saturation with *N*-hydroxytyrosine, D: saturation with *N*-hydroxytyrosine, then titration with tyrosine.

versa does not result in increased absorption in the substrate binding spectrum (Fig. 3) /17/. This demonstrates that tyrosine and *N*-hydroxytyrosine are mutually exclusive substrates that bind to the same active site of cytochrome P450$_{TYR}$.

Earlier data have shown that two molecules of oxygen are consumed in the conversion of tyrosine to *p*-hydroxyphenylacetaldehyde oxime /12/. $^{18}O_2$ labelling experiments have demonstrated that the oxygen atom introduced into the hydroxyamino group of *N*-hydroxytyrosine is lost in the conversion to *p*-hydroxyphenylacetaldehyde oxime, whereas the second oxygen atom introduced in the conversion of *N*-hydroxytyrosine to oxime is 100% retained in the oxime /18/. This indicates that there is no rotation around the C-N bond and that the enzyme converting *N*-hydroxytyrosine to oxime is able to distinguish between the two oxygen atoms introduced /18/. From these data it is concluded that the conversion of tyrosine to *p*-hydroxyphenylacetaldehyde oxime proceeds via two N-hydroxylations of which the first is known to produce *N*-hydroxytyrosine. We now propose that cytochrome P450$_{TYR}$ in the second N-hydroxylation converts *N*-hydroxytyrosine to *N,N*-dihydroxytyrosine, which dehydrates to form 2-nitroso-3-*p*-hydroxyphenylpropionic acid, which is very labile and decarboxylates into *p*-hydroxyphenylacetaldehyde oxime (Fig. 1). Whether the dehydration and decarboxylation reactions are catalyzed enzymatically or proceed spontaneously remains unknown. Generally, cytochromes P450 do not catalyze decarboxylation reactions /19/. In a previous paper, we demonstrated that 2-nitro-3-*p*-hydroxyphenylethane was produced in significant amounts from *N*-hydroxytyrosine and that 2-nitro-3-*p*-hydroxyphenylethane was metabolized into *p*-hydroxymandelonitrile by the microsomal enzyme system /12/. These results combined with the consumption of two molecules of oxygen in the conversion of tyrosine to the oxime made us propose that 2-*aci*-nitro-3-*p*-hydroxyphenylethane was an intermediate between *N*-hydroxytyrosine and the oxime /12/. In light of the new data, we now consider 2-nitro-3-*p*-hydroxyphenylethane a side product of the pathway. One possibility is that 2-nitroso-3-*p*-hydroxyphenylpropionic acid accidentally gets hydroxylated by cytochrome P450$_{TYR}$ *in vitro* resulting in the formation of 2-nitro-3-*p*-hydroxyphenylpropionic acid, which spontaneously decarboxylates to 2-nitro-3-*p*-hydroxyphenylethane. Alternatively, *N,N*-dihydroxytyrosine could become chemically oxidized to the nitro compound. The metabolism of 2-nitro-3-*p*-hydroxyphenylethane to *p*-hydroxymandelo-

nitrile by the microsomal enzyme system demonstrates that the nitro compound re-enters the pathway at a later stage. A dehydrogenation step is required for the formation of the oxime from the nitro compound.

Biosynthetic experiments have demonstrated that the microsomal enzyme system is highly organized exhibiting the phenomenon of catalytic facilitation (channelling), i.e. the enzyme system preferentially metabolizes *in situ* produced intermediates rather than exogenously added intermediates /20/. These data show that both *N*-hydroxytyrosine and *p*-hydroxyphenylacetonitrile are channelled intermediates, whereas (*Z*)-*p*-hydroxyphenylacetaldehyde oxime as the only intermediate in the pathway exchanges freely with exogenously added (*Z*)-*p*-hydroxyphenylacetaldehyde oxime. These results were interpreted to indicate that the biosynthetic pathway for dhurrin is catalyzed either by two highly organized multifunctional enzyme complexes or by two multifunctional enzymes /20/. The identification of cytochrome $P450_{TYR}$ as a multifunctional enzyme catalyzing the conversion of tyrosine to *p*-hydroxyphenylacetaldehyde oxime explains the channelling of *N*-hydroxytyrosine. The present knowledge that the intermediates between tyrosine and *p*-hydroxyphenylacetaldehyde oxime are kept within a single catalytic site raises the question of the true nature of the previously identified or proposed intermediates. Maybe *N*,*N*-dihydroxytyrosine and 2-nitroso-3-(*p*-hydroxyphenyl)-propanoic acid or even *N*-hydroxytyrosine should not be considered genuine intermediates in the pathway. More likely, the true intermediates are short-lived, reactive complexes between different N-oxygenated tyrosine species and cytochrome $P450_{TYR}$. *N*-Hydroxytyrosine may be released in low amounts from these more or less stable transition states. A similar speculation results from studies with the multifunctional cytochrome P-450_{SCC} from adrenal cortex which catalyzes the conversion of cholesterol to pregnenolone /21,22/. 22*R*-Hydroxycholesterol and 20α,22*R*-dihydroxycholesterol are sequentially formed during the conversion. Although hydroxylated and thus more polar compared to cholesterol, they bind more tightly to cytochrome P-450_{SCC} than does cholesterol and they utilise specific interactions of the two side chain hydroxyls with the polypeptide chain. During each catalytic cycle, short-lived, reactive complexes between oxygenated steroidal species and cytochrome P-450_{SCC} are formed. These short-lived complexes or the detected stable hydroxylation products may be subjected to further electron donation and oxygenation.

CLONING AND EXPRESSION OF CYTOCHROME P450$_{TYR}$

Specific polyclonal antibodies towards cytochrome P450$_{TYR}$ and degenerated oligonucleotides derived from amino acid sequences have successfully been used to screen a sorghum cDNA library for a full length cDNA clone encoding cytochrome P450$_{TYR}$ /23/. The isolated and characterized cDNA clone encoding cytochrome P450$_{TYR}$ is among the first five cDNAs of plant cytochromes P450 for which a function can be assigned. The DNA sequence of cytochrome P450$_{TYR}$ shows less than 40% similarity to other known cytochromes P450, and it has therefore been designated the first member of a new family CYP79 /23/.

Heterologous expression of cytochrome P450$_{TYR}$ in *Escherichia coli* has been carried out as described by Barnes *et al.* /24/. The expression vector pSP19g10L was provided by Dr. Henry Barnes, Karolinska Institute (Stockholm, Sweden). The coding region of cytochrome P450$_{TYR}$ was introduced into the expression vector via polymerase chain reaction mutagenesis and transformed into *E. coli*. Upon induction with isopropyl β-D-thiogalactopyranoside (IPTG) *E. coli* produced a functionally active cytochrome P450$_{TYR}$ /25/. Reconstitution experiments using cytochrome P450$_{TYR}$ isolated from *E. coli* membranes and sorghum NADPH-cytochrome P450-reductase demonstrate conclusively that cytochrome P450$_{TYR}$ is multifunctional and catalyses the conversion of tyrosine to *p*-hydroxyphenylacetaldehyde oxime /25/. Administration of ^{14}C-tyrosine directly to *E. coli* cells expressing cytochrome P450$_{TYR}$ results in the production of *p*-hydroxyphenylacetaldehyde oxime, indicating that *E. coli* is able to provide the reducing equivalents for cytochrome P450$_{TYR}$ (Fig. 4).

CONCLUSION

The haem-thiolate *N*-hydroxylase cytochrome P450$_{TYR}$, the committed enzyme in the biosynthesis of the cyanogenic glucoside dhurrin in sorghum, has been purified to homogeneity by dye affinity chromatography. Reconstitution of cytochrome P450$_{TYR}$ monooxygenase activity has demonstrated that cytochrome P450$_{TYR}$ is multifunctional catalyzing two consecutive N-hydroxylations resulting in the conversion of tyrosine to *p*-hydroxyphenylacetaldehyde oxime. In animal steroid biosynthesis there are at least four examples of purified cytochromes P450 which catalyze two or three successive and

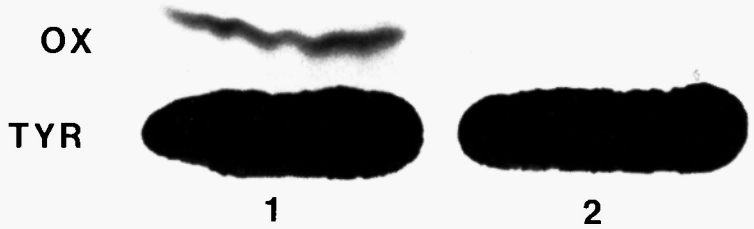

Fig. 4: Autoradiogram of TLC chromatogram of *E. coli* cells incubated with ^{14}C-tyrosine. 1. *E. coli* transformed with the pSP19g10L plasmid containing the native sequence of cytochrome $P450_{TYR}$; 2. *E. coli* transformed with the pSP19g10L plasmid.

coordinated oxygen activation and oxygen insertion reactions at their active sites /24/. Cytochrome $P450_{TYR}$ is the first known multi-functional cytochrome P450 from plants. N-Hydroxylases of the cytochrome P450-type with high substrate specificity have not previously been reported.

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