Localization of Cinnamic Acid 4-Monoxygenase and the Membrane-bound Enzyme System for Dhurrin Biosynthesis in Sorghum Seedlings\textsuperscript{1}

Received for publication May 11, 1977 and in revised form June 27, 1977

JAMES A. SAUNDERS, ERIC E. CONN, CHIN HO LIN,\textsuperscript{2} AND MIKIO SHIMADA\textsuperscript{3}
Department of Biochemistry and Biophysics, University of California, Davis, California 95616

ABSTRACT

The localization of three monoxygenase (hydroxylase) enzyme systems which occur in dark-grown seedlings of Sorghum bicolor has been studied. Cinnamic acid 4-hydroxylase (CAH) (trans-cinnamate 4-monoxygenase, EC 1.14.13.11), which has been increasingly utilized in plants as a marker for the endoplasmic reticulum, migrated with that fraction in continuous and discontinuous sucrose gradients. When 10 mM MgCl\textsubscript{2} was used to shift the density banding of the marker enzyme, NADPH cytochrome c reductase, from 1.12 to 1.17 g/cm\textsuperscript{3}, the CAH activity was displaced as well.

The membrane-bound enzyme system involved in the biosynthesis of the cyanogenic glucoside dhurrin was also shown to be closely associated with the endoplasmic reticulum. This system contains hydroxylases capable of hydroxylating tyrosine to form N-hydroxytyrosine and hydroxylating p-hydroxyphenylacetonitrile to form p-hydroxy-(S)-mandelonitrile.

Cinnamic acid 4-hydroxylase (EC 1.14.13.11), which catalyzes the formation of p-coumaric acid from trans-cinnamic acid, is a key enzyme in the synthesis of numerous compounds in what is termed the general phenylpropanoid pathway. This enzyme was first detected in microsomal membranes of pea seedlings by Russell and Conn (17) and has subsequently been carefully characterized in Sorghum microsomes (13). Although not of homogeneous consistency, the microsomal system has provided a convenient source of the enzyme for a number of workers (13, 15–17, 23, 24, 26).

Tanaka \textit{et al.} (23, 24) have further examined the microsomal membranes of Brassica and proposed that the CAH\textsuperscript{4} activity is not associated with ER although that membrane is an important component of the microsomal fraction. In contrast, Young and Beevers (26) have concluded that, in Ricinus endosperm tissue, CAH activity is principally associated with the ER, although the rates of activity observed were low. Recent studies by Benveniste \textit{et al.} (3) have supported the proposal that the CAH activity is associated with the ER.

\textit{Sorghum} microsomal membranes also contain enzymes capable of synthesizing intermediates in the biosynthesis of the cyanogenic glucoside dhurrin (10). Two of these are monoxygenases (hydroxylases) which convert tyrosine to N-hydroxytyrosine\textsuperscript{5} and p-hydroxyphenylacetonitrile to p-hydroxymandelonitrile (20). The present investigation was undertaken to determine not only if the CAH of Sorghum is associated with the ER, but also to obtain information on the localization of the membrane-bound enzyme system involved in dhurrin biosynthesis.

MATERIALS AND METHODS

\textbf{Plant Material.} Fruits of \textit{Sorghum bicolor} (Linn) Moench, variety Sordan 70 obtained from Northrup King and Company, Lubbock, Texas, were soaked in aerated water for 24 hr and germinated in the dark at 25 C for 2 days on cotton gauze saturated with water. Etiolated seedlings grown in this manner were used in all subsequent experiments except for those involving Chl measurements, in which case the seedlings were grown for 5 days under a 14-hr photoperiod.

\textbf{Preparation of Microsomal Pellet.} Approximately 15 g of seedlings were harvested by excision of the 3-cm shoot 1 cm above the caryopsis and grinding in a chilled mortar and pestle. The basic homogenizing medium consisted of 0.3 M sucrose, 25 mM tris-HCl buffer (pH 8), 1 mM DTT, and 0.1 g insoluble PVPP/g of tissue fresh wt. This medium was supplemented with the addition of either 10 mM MgCl\textsubscript{2} or 10 mM EDTA disodium salt for specific experimental requirements. The homogenate was filtered first through two layers of cheesecloth and then two layers of Miracloth; the filtrate was then subjected to differential centrifugation at 5,000g for 10 min, 20,000g for 30 min, and 100,000g for 1 hr.

\textbf{Sucrose Gradient Centrifugation.} Continuous sucrose gradients were prepared from 15 and 55\% (w/w) sucrose stock solutions containing 25 mM tris-HCl buffer (pH 8), with or without 10 mM MgCl\textsubscript{2}, using a multiple sucrose gradient maker (Hoefer Scientific). Five ml of the post-5,000g supernatant fraction was layered onto the gradient and centrifuged at 26,000 rpm at 2 C in a Beckman SW 27 swinging bucket rotor. As the presence of 10 mM MgCl\textsubscript{2} in the gradient facilitated equilibrium banding of the ER, those gradients with Mg were centrifuged for 4 hr while those without Mg were centrifuged for 10 hr in a Sorvall OTD-50 ultracentrifuge. The gradient fractions were collected by puncturing the cellulose nitrate tubes from below, pumping in 65\% (w/w) sucrose, and collecting 1-ml fractions from the top of the gradient with a Gilson mini-escargot fraction collector.

Discontinuous sucrose gradient centrifugation was accom-

\textsuperscript{1} This work was supported in part by National Science Foundation Grant BMS 74-11997-A01 and United States Public Health Service Grant GM 05301-19 to E. E. C.

\textsuperscript{2} Present address: Botany Department, National Chung Hsing University, Taiwan.

\textsuperscript{3} Present address: Wood Research Institute, Kyoto University, Uji, Japan.

\textsuperscript{4} Abbreviation: CAH: cinnamic acid 4-hydroxylase.

\textsuperscript{5} B. L. Møller and E. E. Conn, unpublished results.
plished by layering the microsomal pellet obtained from the 100,000g centrifugation onto a preformed gradient of 25 mm tris-HCl buffer (pH 8), and 10 mm MgCl₂ which contained bands of 27, 34, and 43% (w/w) sucrose. The gradient was then centrifuged at 26,000 rpm for 2 hr in a SW 27 Beckman rotor for 4 hr and the layers at the interfaces collected with a 2-ml syringe.

**Enzyme Assays.** NADPH-Cyt-c reductase was selected as the ER marker enzyme and assayed by a modification of the procedure of Williams and Kamin (25). The reaction mixture consisted of 100 nmol of Cyt c, 1 mm sodium cyanide, 20 to 80 μg of protein, and 1.5 ml of 50 mm K-phosphate buffer (pH 7.7) in a total volume of 2 ml. The reaction was started by the addition of 200 nmol of NADPH and the change in A was recorded at 550 nm for 2 min. The addition of 50 nmol of antimycin A to the reaction mixture had little effect (less than 10%) on the rate of the reaction.

Cinnamic acid 4-hydroxylase was measured by the technique of Potts et al. (13) modified to include a thin layer separation of the reactant and product in benzene-glacial acetic acid (4:1). The cpm of p-coumaric acid and cinnamic acid were normalized for variations of total activity among samples.

The mitochondrial marker enzyme succinic acid dehydrogenase was assayed by the method of Pennington (12).

The membrane-bound enzyme system involved in the biosynthesis of dhurrin was studied by two different procedures. In procedure A, the C-hydroxylation of p-hydroxyphenylacetonitrile by the enzyme designated as nitrile hydroxylase was measured by determining the HCN produced on dissociation of the p-hydroxymandelonitrile formed (10). p-Hydroxyphenylacetaldoxime (aldoxime) was used as a substrate because it is readily dehydrated by Sorghum microsomes to form p-hydroxyphenylacetonitrile (nitrile); the nitrile produced in situ by this procedure is utilized more effectively as a substrate than nitrile added exogenously (20). The reaction mixture (500 μl) by this procedure contained approximately 50 μg of protein, 3 μmol of glucose-6-P, 0.3 μmol of NADP⁺, 4 units of glucose-6-P dehydrogenase, 250 μl of 0.3 M Tricine buffer (pH 8). 1.4 μl of 20 mm mercaptoethanol, and 1 μmol of p-hydroxyphenylacetaldoxime. The solution was incubated for 2 hr at 30 C in a small capped vial fitted with a cap. A solution containing 300 μl 1 N NaOH suspended above the reaction mixture. The reaction was terminated by injection of 50 μl of 3 N H₂SO₄ and the sample incubated overnight with shaking to trap released cyanide. The cyanide was quantitated by the technique of Epstein (4).

In procedure B, the conversion of l-tyrosine to p-hydroxymandelonitrile by the multistep sequence of dhurrin biosynthesis was observed. This process, termed the tyrosine-metabolizing system, involves both the C-hydroxylation of p-hydroxyphenylacetonitrile measured in procedure A, and the N-hydroxylation of l-tyrosine. The tyrosine-metabolizing system is measured by determining the amount of p-hydroxybenzaldehyde formed on dissociation of p-hydroxymandelonitrile. During the conversion of tyrosine to p-hydroxymandelonitrile, p-hydroxyphenylacetaldoxime accumulates to some extent in the reaction mixture and can be measured (10, 20). When this is done, only the initial reactions of the tyrosine-metabolizing system are measured, one of which is the N-hydroxylation of tyrosine. The reaction mixture (600 μl) consisted of 3 μmol of glucose-6-P, 0.3 μmol of NADP⁺, 4 units of glucose-6-P dehydrogenase, 250 μl of 0.3 M Tricine buffer (pH 7.2), 1 mm DTT, and 50 μg of protein. The reaction was started by the addition of 50 μl of a solution containing 40 μCi/ml of L-[3,5-³H]tyrosine (42 Ci/mm) and 2.2 μg/ml of unlabeled tyrosine. The solution was incubated at 30 C for 2 hr and terminated by the addition of 100 μl of 3 N H₂SO₄. An aliquot was chromatographed on Silica Gel IB-F thin layer plates in benzene-ethyl acetate (5:1), to separate p-hydroxybenzaldehyde from the unreacted tyrosine and the p-hydroxyphenylacetaldoxime that accumulates. These compounds were cut out of the thin layer chromatogram and the pieces added to scintillation vials for counting.

Radioactivity was determined in a Beckman scintillation counter by adding 1 ml of water to solubilize the compounds followed by 10 ml of Triton-toluene mixture composed of 500 ml Triton X-100, 1 liter of toluene, 0.5% PPO, and 0.02% POPPOP.

Chl was determined by the method of Arnon (2) and protein by the sulfohalic acid turbidometric method discussed by Layne (7). Densities were determined with a Bausch and Lomb desk model refractometer.

**Electron Microscopy.** Membrane fractions were fixed in 5% glutaraldehyde in 50 mm cacodylate buffer (pH 7.1) for 15 min, rinsed with distilled H₂O, and dehydrated in 2,2-dimethoxypropane for 30 min by a technique described elsewhere (18).

**Chemicals.** Reagents for the various assays were obtained from Sigma Chemical Co. L-[3,5-³H]Tyrosine (42 Ci/mm) was obtained from Amersham/Searle and [2-¹⁴C]cinnamic acid (4 mCi/mm) was obtained from ICN Isotope and Nuclear Division. All other chemicals were of reagent grade or better.

**RESULTS**

The biosynthesis of dhurrin in *Sorghum* seedlings involves a membrane-bound enzyme system which converts tyrosine to p-hydroxy(S)mandelonitrile by a multistep pathway (10). In the presence of UDP-glucose and a soluble glucosyltransferase (14), the p-hydroxymandelonitrile is converted to dhurrin (10). The membrane-bound enzyme system, which may be followed by its ability to catalyze the conversion of tyrosine, p-hydroxyphenylacetaldoxime, or p-hydroxyphenylacetonitrile to p-hydroxymandelonitrile, is located in the microsomal fraction obtained on homogenization of the young seedlings (10, 20). Typical data, not previously reported, are shown in Table I where 52% of the enzyme system, as measured by its nitrile hydroxylase (procedure A) activity, is shown in the 100,000g pellet obtained on differential centrifugation of a homogenate of etiolated *Sorghum* shoots.

Table I also presents data on the specific activities of CAH, NADPH-Cyt-c reductase, and succinic acid dehydrogenase in the fractions obtained on differential centrifugation together with the Chl concentration and total protein content. The fractions with the two highest specific activities for succinic acid dehydrogenase were the 5,000g and 20,000g pellets; these contained 69% of the total enzyme activity. On the other hand, the highest specific activity (and the largest fraction of the total activity) for CAH and for NADPH-Cyt c reductase was observed in the particulate fraction obtained from the 100,000g centrifugation. The similar distribution of CAH and NADPH-Cyt c reductase (correlation coefficient of r = 0.961) in this pellet suggested that these two enzyme systems occur in the same membrane fraction.

In an attempt to refine the system further, the post-5,000g supernatant fraction was examined by continuous sucrose gradient centrifugation. Figure 1 illustrates a sucrose gradient from 15 to 50% (w/w) sucrose with 10 mm MgCl₂. Under these conditions the NADPH-Cyt c reductase and CAH activities comigrated in the gradient to a density of 1.16 to 1.17 (fractions 29-34). Succinate dehydrogenase activity equilibrated at a higher density (fraction 40) while the soluble protein remained in the less dense portion of the gradient (fractions 9-13). The nitrile hydroxylase activity was concentrated in fractions 34 and 35 in the same gradient; that is, it was slightly shifted to the right of the main band of CAH and NADPH-Cyt c reductase activities. Such distribution indicates the association of the tyrosine-metabolizing system with the microsomal fraction though not necessarily with the ER. The large shoulder of
Table 1. The Distribution of Some Particulate Enzymes in Homogenized Sorghum Seedlings

<table>
<thead>
<tr>
<th>Protein</th>
<th>Chlorophyll</th>
<th>Cinnamic Hydroxylase</th>
<th>NADPH-Cyt c Reductase</th>
<th>Nitrile Hydrolase</th>
<th>Succinate Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td>(mg/g)</td>
<td>(mg/g)</td>
<td>(mg/g)</td>
<td>(mg/g)</td>
</tr>
<tr>
<td></td>
<td>(total)</td>
<td>(total)</td>
<td>(total)</td>
<td>(total)</td>
<td>(total)</td>
</tr>
<tr>
<td>5,000 g</td>
<td>95</td>
<td>110.7</td>
<td>2,065</td>
<td>0.045</td>
<td>0.484</td>
</tr>
<tr>
<td>pellet</td>
<td>(18%)</td>
<td>(32%)</td>
<td>(9%)</td>
<td>(11%)</td>
<td>(23%)</td>
</tr>
<tr>
<td>20,000 g</td>
<td>80</td>
<td>186.3</td>
<td>7,602</td>
<td>0.114</td>
<td>8.4</td>
</tr>
<tr>
<td>pellet</td>
<td>(15%)</td>
<td>(55%)</td>
<td>(28%)</td>
<td>(23%)</td>
<td>(17%)</td>
</tr>
<tr>
<td>100,000 g</td>
<td>110</td>
<td>42.5</td>
<td>12,308</td>
<td>0.239</td>
<td>18.2</td>
</tr>
<tr>
<td>pellet</td>
<td>(21%)</td>
<td>(12%)</td>
<td>(62%)</td>
<td>(66%)</td>
<td>(52%)</td>
</tr>
<tr>
<td>100,000 g</td>
<td>231</td>
<td>2.3</td>
<td>0</td>
<td>5.0</td>
<td>0.060</td>
</tr>
<tr>
<td>supernatant</td>
<td>(45%)</td>
<td>(0.72%)</td>
<td>(0%)</td>
<td>(30%)</td>
<td>(13%)</td>
</tr>
</tbody>
</table>

Fig. 1. Continuous sucrose gradient centrifugation of a Sorghum homogenate with 10 mM MgCl₂ centrifuged for 4 hr at 110,000 g. (---): cinnamic acid 4-hydroxylase; (Δ--Δ): succinic acid dehydrogenase; (O--O): tyrosine-metabolizing system as measured by cyanide production or procedure A; (O----O): density g/cm³; (Δ--Δ): protein.

membranes associated with the 20,000g mitochondrial pellet and 100,000g microsomal pellet, respectively. These figures show that the microsomal pellet is based on a procedure containing the step of sucrose addition and is not composed of homogeneous cytoplasmic organelles. The mitochondrial pellet contains several membrane types but is enriched in mitochondria; however, relatively few ribosomes are seen.

The presence of Mg²⁺ in the homogenization medium and sucrose gradient exhibited a dramatic effect on the equilibrium banding pattern of the ER because of its action on the dissociation of ribosomes (9). Therefore, by omitting MgCl₂, the ER could be shifted from a density of 1.17 to 1.12 as shown in Figure 3. Under these conditions, the CAH activity co-migrated precisely with NADPH-Cyt c reductase in fractions 9 to 14 while the succinate dehydrogenase activity (fractions 23–27) remained relatively unchanged in its banding pattern (Fig. 3). In the absence of MgCl₂, the enzyme system associated with the biosynthesis of dhurrin has also shifted to a lower density. When procedure B was used to measure the tyrosine-metabolizing system, the activity coincided with the ER (fractions 10–13). When the partial conversion of tyrosine to p-hydroxyphenylacetaldimine was measured the peak of activity was observed in two fractions to the right of the main band of ER.

In a further attempt to determine whether the enzyme system associated with dhurrin biosynthesis resides in the ER, the microsomal pellet obtained from a 100,000g centrifugation (Table I) was subjected to discontinuous sucrose gradient centrifugation in the presence of 10 mM MgCl₂. The results again showed (Fig. 4) that the distribution of CAH activity correlated well with that of NADPH-Cyt c reductase activity. Correlation statistics indicated a high degree of positive correlation (r = 0.969) in the four fractions obtained. On the other hand the enzymes of dhurrin biosynthesis (as measured by procedure B) were located at a lower portion of the gradient than the other two enzymes. Correlation statistics for the tyrosine-metabolizing system assay versus NADPH-Cyt c reductase specific activity yielded a median value of r = 0.602.

Figures 2, C and D are photomicrographs of the membranes associated with the bands at the 27/34% and 34/43% (w/w) sucrose interfaces, respectively. Note that the upper band appears to have a higher concentration of free ribosomes while the lower band contains recognizable RER.
Fig. 2. Membrane fractions from a homogenate of *Sorghum* seedlings. A: Pellet obtained from differential centrifugation of the homogenate at 20,000g for 30 min showing mitochondrial enrichment of the membranes (× 29,000); B: microsomal pellet obtained upon centrifugation of the postmitochondrial pellet at 100,000g for 1 hr indicating the heterogeneous nature of this fraction (× 29,900); C: discontinuous gradient fraction accumulating at the 25/33% (w/w) sucrose interface during fractionation of the microsomal pellet (× 29,600); D: discontinuous gradient fraction accumulating at the 33/43% (w/w) sucrose interface during further fractionation of the microsomal pellet (× 34,000).
Sorghum seedlings their external dehydrogenase; acid involve a homogenate with 10 NADPH Cyt c reductase; NADPH: cytochrome c reductase; TMS: tyrosine-metabolizing system measured by procedure A. Correlation coefficient for CAH:CCR and CCR:TMS are given.

In preliminary work (13), this laboratory reported the occurrence of the enzyme in a light membrane fraction with a density of 1.12 to 1.14 g/cm^3. Tanaka et al. (23, 24), in a study of the injury-induced CAH of sweet potato tuber that involved comparison with marker enzymes of mitochondria and ER, concluded that CAH was not distributed, in either continuous or discontinuous gradients, in a manner identical with NADPH-Cyt c reductase. It should be noted, however, that the sucrose gradients examined by these workers exhibited quite broad bands of activities for the ER marker enzymes. Our own data suggest that extended centrifugation times are needed for tight equilibrium banding in the absence of MgCl_2. The broad distribution of activity of the marker enzymes may have led Tanaka et al. to an erroneous interpretation of the actual localization. Alternatively, the results obtained by Tanaka et al. may represent a real difference characteristic of the wound-induced CAH of sweet potato.

In recent work with well equilibrated, continuous sucrose gradients, Young and Beever (26) have shown that the CAH of Ricinus endosperm tissue is mainly associated with the ER with minor amounts aligned with the mitochondrial and glyoxysomal fractions. Similarly, Benveniste et al. (3) have recently concluded that the injury-induced CAH of Jerusalem artichoke tuber is localized on the ER of that tissue, and Rich and Lamb (15) assigned the slicing-induced CAH of potato tubers to the smooth membranous vesicles (Buoyant density 1.11-1.14 g/cm^3) which contain Cyt P-500. Our data in the present paper on the cellular localization of CAH of Sorghum seedlings confirm the conclusion of Young and Beever (26) and of Benveniste et al. (3) that the enzyme is located on the ER. The use of CAH as a marker enzyme for the ER, as is now being done (6), therefore, appears justified.

The careful work with endosperm of castor bean was facilitated by the thorough characterization of the cellular organelles of that tissue carried out over a period of years (26). The level of CAH in the isolated Ricinus ER fraction was almost two orders of magnitude lower than that in Sorghum tissue. Our continuing interest in the enzymes of phenylpropanoid metabolism therefore made it desirable to locate the CAH of Sorghum which is comparatively enriched in this enzyme activity. Another reason for determining the cellular localization of the Sorghum CAH is that it appears to be a constitutive enzyme in this tissue in contrast to the enzyme in Ipomoea, Solanum, and Helianthus where the enzyme is induced by injury. A third reason was to compare the relationship, if any, of CAH to the highly active membrane-bound enzyme system in Sorghum involved in the biosynthesis of dhurrin (10).

The dhurrin-synthesizing system, which like the CAH is highly substrate-specific, involves two monooxygenation reactions that to date have not been shown to involve Cyt P-500.
However, the dhurrin-synthesizing system has a distribution pattern very similar to that of the ER marker enzyme, NADPH-Cyt c reductase, and therefore of CAH. Upon the addition of 10 mM MgCl₂, the dhurrin-synthesizing system, as well as CAH, is shifted from a density of 1.12 to 1.17. This shift is attributed to the dissociation of ribosomes from membranous systems and is characteristic of ER (9). Difficulties inherent in the quantitation of a multistep reaction sequence require that caution be exercised in making final conclusions on this point. Nevertheless, it is noteworthy that the ER of Sorghum seedlings possesses three highly reactive hydroxylation systems involved in the biosynthesis of natural products.

The association of CAH, the second enzyme in the phenylpropanoid pathway, with the ER raises interesting logistical questions regarding the biosynthesis of the phenylpropanoids, especially since phenylalanine ammonia lyase, the first enzyme in the pathway, is generally regarded as a soluble enzyme. Stafford, in discussions of this problem (22), has proposed that phenylalanine ammonia lyase and CAH may occur, possibly together with other enzymes required to specific biosynthetic sequences, in multienzyme complexes. Such complexes may be "attached to cytoplasmic membranes or enclosed within organelles." The reported occurrence of phenylalanine ammonia lyase in microsomes (1), glyoxysomes (5), and chloroplasts (8, 11, 19) suggests that these enzyme complexes may involve more than one organelle.

Recent studies by Saunders et al. (18) have presented data showing that the Sorghum cyanogenic glucoside is accumulated in the vacuole based on a microautoradiographic study. Attempts are now underway to characterize the tonoplast membrane in order to determine if it possesses any of the enzymes for the biosynthesis of the cyanogenic glucoside of Sorghum.

Acknowledgments—The authors would like to express their appreciation to C. R. Stocking for the use of the electron microscope facilities and to N. B. Saunders for the skillful technical assistance when needed.

LITERATURE CITED

1. Amrein N, MH Zunk 1970 Concomitant induction of phenylalanine ammonia-lyase and cinnamic acid 4-hydroxylase during illumination of excised buckwheat hypocotyls. Naturwissenschaften 57: 312
17. Russell DW, EE Conn 1967 The cinnamic acid 4-hydroxylase of pea seedlings. Arch Biochem Biophys 122: 256-258