EVIDENCE FOR AN ELECTROPHILIC INTERMEDIATE IN THE MICROSOMAL HYDROXYLATION OF CINNAMIC ACID IN PLANTS

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1. Introduction

The 4-hydroxylation of cinnamic acid is catalyzed by a plant microsomal mixed-function oxygenase system which resembles the liver microsomal mixed-function oxygenase system by a number of criteria, viz., participation of cytochrome *P*-450, inducibility, dependence upon a lipid cofactor, sensitivity to characteristic inhibitors and a 1,2-('NIH')-shift of hydrogen (reviewed [1]). The NIH-shift is usually taken as an indication for an electrophilic epoxide intermediate [2,3]. Naphthalene 1,2-epoxide has, in fact, been isolated as an intermediate of the liver microsomal hydroxylation of naphthalene [4]. However, an aryl epoxide intermediate has so far not been demonstrated for plant microsomal hydroxylation reactions. Because of the extreme lability of monocyclic aromatic epoxides [3], the nucleophilic agent, glutathione, has now been employed to trap a presumed electrophilic intermediate of cinnamic acid hydroxylation. Some of the results have been briefly communicated [1].

2. Experimental

The isolation of microsomal fractions by Mg^{2+} precipitation, the incubation procedures, and the determinations of total membrane protein and of radioactivity have been described [5,6]. Solvent systems used for descending paper chromatography were:

(A) benzene/acetic acid/water, 2:1:1 (v/v/v; upper phase);
(B) butanol-1/acetic acid/water, 2:1:1 (v/v/v);
(C) water/acetic acid, 98:2 (v/v).

Abbreviations: GSH, glutathione; GSSG, oxidised glutathione

3. Results and discussion

3.1. *Effects of glutathione*

Glutathione (5 mM) was added to the standard cinnamic acid 4-hydroxylase incubation mixture, in the presence or absence of 1 mM NADPH or 2.5 mM β-mercaptoethanol, respectively (table 1). The amounts of *p*-coumaric acid (R_F 0.3) and of polar products (R_F below 0.1) were determined by use of solvent system (A). Duplicate samples were chromatographed in solvent system (B), in order to determine the amount of glutathione conjugates (R_F 0.4–0.6). In the presence of glutathione, the amount of *p*-coumaric acid formed was decreased. Polar products which apppeared to consist largely of glutathione conjugates were detected. However, glutathione conjugates were also formed in the absence of NADPH which was required for the formation of *p*-coumaric acid. An explanation of these observations was only provided by chromatographic analysis (fig.1). Two glutathione conjugate products appeared to be formed in the complete incubation mixture. Their R_F-values were 0.4 and about 0.5, respectively (fig.1, curve A). When heat-inactivated enzymes was employed, only a small amount of the R_F 0.5 conjugate was detected (fig.1, curve B). When the microsomal fraction of dark-grown parsley cells was employed, only the product migrating at R_F 0.5 was

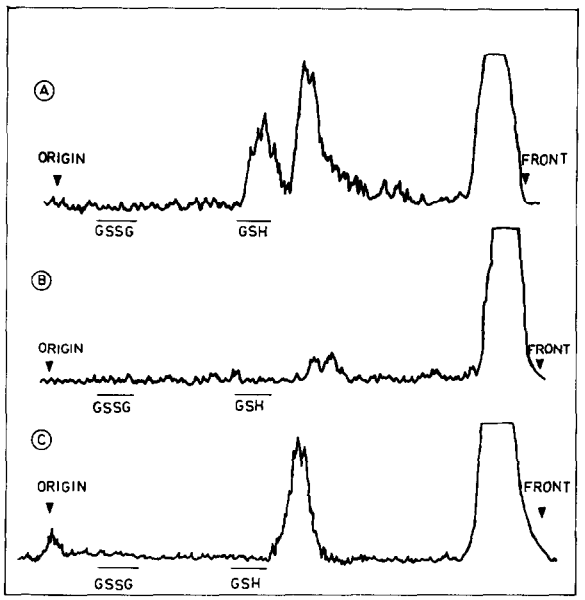

Fig.1. Chromatographic detection of glutathione *S*-cinnamoyl conjugates. Curve A: The incubation mixture contained 0.5 mM [3-^{14}C]cinnamic acid (0.1 μCi), 1 mM NADPH, 3 mM glutathione, 100 mM Tris–HCl (pH 7.5) and 250 μg microsomal protein from light-treated parsley cells in total vol. 125 μl. After incubation for 60 min at 30°C, the incubation mixture was applied to paper for development in solvent system (B). Curve B: Same incubation as for curve A. The microsomal fraction was heat-denatured prior to the assay (15 min, 100°C). Curve C: Same incubation as for curve A. The microsomal fraction was isolated from dark-grown parsley cells.

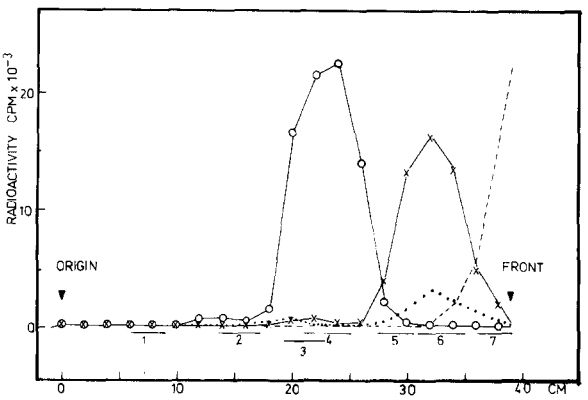

Fig.2. Desulfurization of glutathione *S*-cinnamoyl conjugates with Raney nickel. The procedure in [17] was slightly modified. The adduct of R_F 0.4 or 0.5, respectively (29 nmol, 80 000 cpm) was lyophilized, and 100 mg Raney nickel (Fluka No. 83440) was added in 5 ml methanol/water, 1:1 (v/v). After refluxing for 2 h at about 80°C, Raney nickel was removed by filtration, and the solutions were chromatographed in solvent system (C). (o—o) Treatment of the R_F 0.4 conjugate. (x—x) Treatment of the R_F 0.5 conjugate. (· · · ·) Treatment of the conjugate fraction formed by heat-denatured microsomes (artifact). (—) Untreated R_F 0.4 and R_F 0.5 conjugates. The positions of the following reference compounds are indicated: (1) caffeic acid; (2) *o,m,p*-coumaric acids; (3) dihydro *p*-coumaric acid; (4) cinnamic acid; (5) cinnamoyl alcohol; (6) dihydrocinnamic acid, phenylpyruvic acid, phenyllactic acid; (7) glutathione.

formed (fig.1, curve C). It therefore appeared that only the R_F 0.4 product was related to cinnamic acid 4-hydroxylase activity which is known to be induced by light [7,8]. Neither of the conjugates was formed when the soluble enzyme fraction of parsley cells was employed, or when dihydrocinnamic acid rather than cinnamic acid was used as the substrate (c.f.[9]).

3.2. *Characterization of the conjugate migrating at* R_F *0.5*

The product migrating at R_F 0.5 (solvent system B) was prepared chromatographically using the microsomal fraction of light-treated parsley cells in the absence of NADPH and β-mercaptoethanol. The product was resistant to treatment with potassium hexacyanoferrate [10] or hydroxylamine [11], indicating that the –SH group of glutathione was substituted, and that no thioester of cinnamic acid had been isolated. Desulfurization with Raney nickel resulted in the release of dihydrocinnamic acid (fig.2). The product of desulfurization also migrated like authentic dihydrocinnamic acid upon rechromatography in solvent system (A) (R_F 0.95; *p*-coumaric acid 0.35; phenyllactic acid 0.54; phenylpyruvic acid 0.66). This result strongly indicated the presence of a thioether bond between glutathione and the olefinic double bond of cinnamic acid (cf. [12]), due apparently to a novel plant microsomal glutathione *S*-alkene transferase activity.

3.3. *Characterization of the conjugate migrating at* R_F *0.4*

The product migrating at R_F 0.4 (solvent system B) was prepared chromatographically using the micro-

somal fraction of light-treated parsley cells in the presence of NADPH and β-mercaptoethanol. The product was resistant to treatment with potassium hexacyanoferrate or hydroxylamine (see above). Desulfurization with Raney nickel resulted in the release of cinnamic acid (fig.2). The product of desulfurization also migrated like authentic cinnamic acid upon rechromatography in solvent system (A) (R_F 0.95; see above). A small amount of coumaric acid (identified in solvent system (A)) was released upon treatment of the R_f 0.4 conjugate with 0.1 N HCl (5 min, 40°C, air atmosphere; 10% yield; see [4]). The results indicate that the R_F 0.4 conjugate contained a thioether bond between glutathione and the aromatic ring of cinnamic acid (cf. [4,12]).

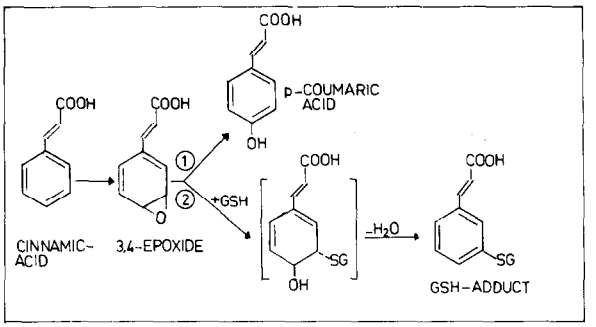

Fig.3. Proposed reaction pathways for the 4-hydroxylation and glutathione conjugation of cinnamic acid. Only the NADPH-dependent reactions of microsomes from light-treated parsley cells are considered. For explanation, see text.

3.4. *Proposed reaction mechanism*

The above results are consistent with the reaction pathway shown in fig.3. The cinnamic acid 4-hydroxylase reaction is thought to proceed via the electrophilic cinnamic acid 3,4-epoxide (pathway 1). In the presence of glutathione, a 3,4-dihydro-3-(or 4-)-hydroxy *S*-cinnamoyl adduct is formed (pathway 2). Elimination of water leads to the final glutathione *S*-cinnamoyl adduct shown (or its 4-isomer).

Aromatic epoxides are known to be central intermediates of detoxication as well as of chemical carcinogenesis in mammalian cells [13]. Plant cells are able to hydroxylate a variety of xenobiotics [14], but very little is known about the hydroxylase systems involved [1]. More specifically, the parsley cell suspension cultures used in the present study have been shown to form polar metabolites from DTT and kelthane [15] and benzo(α)pyrene [16]. The present results suggest that the conjugation of epoxide intermediates may possible constitute a new way for the detoxication of xenobiotics by plants.

Table 1
Products formed by the microsomal fraction of parsley cells from [3-^{14}C]cinnamic acid

Amount of product (nmol/mg protein)	Component added to the basic incubation mixture containing buffer system (a) or (b)							
	None		NADPH		GSH		NADPH + GSH	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
p-Coumaric acid	0.3	0.4	18.8	12.0	0.2	0.1	13.7	3.6
Total polar products	0	0	0.8	3.2	17.9	2.4	5.9	7.6
Glutathione conjugates	0	0	0.4	0.3	16.1	4.4	6.3	5.2

The microsomal fraction was isolated from light-treated parsley cells in the presence of 2.5 mM β-mercaptoethanol [6]. The microsomal fraction was divided into two portions which were suspended in either (a) 100 mM Tris-HCl (pH 7.5) or (b) 100 mM Tris–HCl, (pH 7.5), containing 2.5 mM β-mercaptoethanol. The incubation mixtures contained 0.5 mM [3-^{14}C]cinnamic acid (0.1 μCi) and, where indicated, 1 mM NADPH or 5 mM glutathione (buffer system (a) or (b), 250 μg protein; total vol. 125 μl). After incubation for 60 min at 30°C, the entire reaction mixtures were applied to paper for development in solvent systems (A) or (B) (see text). The amounts of product shown were corrected for non-enzymatic reaction by taking into account the amounts of product formed by heat-denatured enzyme (15 min, 100°C)

Acknowledgements

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