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Aldoxime-Forming Microsomal Enzyme Systems Involved in the Biosynthesis of Glucosinolates in Oilseed Rape (*Brassica napus*) Leaves[1]

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Glucosinolates and cyanogenic glucosides are synthesized from amino acids via similar intermediates, *N*-hydroxyamino acids and aldoximes. Microsomal preparations from young green leaves of oilseed rape catalyze the NADPH-dependent metabolism of homophenylalanine and dihomomethionine to the respective aldoximes, precursors of 2-phenylethyl and 3-butenyl glucosinolates. Cytochrome P-450-type enzymes are not involved (in contrast to cyanogenic glucoside biosynthesis), because neither activity was affected by carbon monoxide or other cytochrome P-450 inhibitors. Copper ions and diethyl pyrocarbonate were potent inhibitors of the enzymes, and treatment of microsomes with detergents abolished the overall activity. Two distinct enzyme systems with similar properties appear to be involved, each specific for a particular substrate. One utilizes dihomomethionine and is not active with homophenylalanine or any other amino acid tested, and the other is specific for homophenylalanine. From the characteristics of these enzymes, it seems that these early steps in glucosinolate biosynthesis may be catalyzed by flavin-containing monooxygenases comparable to those found in mammalian tissues and elsewhere. The pathways for the biosynthesis of glucosinolates and cyanogenic glucosides have apparently evolved independently, despite the similar chemical conversions involved.

The glucosinolates are a class of N- and S-containing secondary metabolites found in the Cruciferae and several other families of dicotyledonous angiosperms. Upon tissue disruption, the enzyme myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1) catalyzes the hydrolysis of glucosinolates to isothiocyanates and other noxious and toxic products (for reviews, see Underhill, 1980; Larsen, 1981; Chew, 1988). Glucosinolates are involved in the interaction of crucifers with herbivores, pests, and pathogens and may play a role in S and N storage within vegetative and seed tissues.

In oilseed rape (*Brassica napus* L.), a range of different glucosinolates are accumulated, depending on the tissue and age (Milford et al., 1989) and the nutritional and stress status of the plant. Mechanical damage, insect feeding, or fungal infection stimulate glucosinolate synthesis and accumulation (Doughty et al., 1991; Koritsas et al., 1991). Three classes of glucosinolates are found in rape, the alkenyl compounds derived from Met, aromatic glucosinolates derived from Phe, and indolylglucosinolates from Trp. For the first two classes, glucosinolates derived from the parent protein amino acid are not present, but, instead, chain-extended homologs appear to be the immediate precursors.

Insect pests that feed or reproduce on brassicas have specific receptors to identify glucosinolate metabolites, and some coleopterous pests of rape recognize a limited range of isothiocyanates derived from alkenyl glucosinolates (Blight et al., 1989). Changing the profile of glucosinolates accumulated could well disrupt host-plant recognition by such pests, without reducing the deterrent effect of glucosinolates on nonspecialist feeders. Similarly, elimination of certain specific glucosinolates from seeds might improve the nutritional value of seed meal, without the increased vulnerability of seeds and emerging seedlings observed with current low-glucosinolate varieties (Glen et al., 1990). Strategies to manipulate the glucosinolate content of oilseed rape or other brassica crops to improve nutritional qualities or natural crop protection depend on a clear understanding of the biosynthetic pathways for these compounds.

Very little is known of the enzymology of glucosinolate biosynthesis; most of our knowledge of the postulated pathway (Fig. 1A) comes from feeding experiments with whole plants or excised tissues (for review, see Moller and Poulton, 1993). Only the last two steps, catalyzed by a glucosyltransferase and a sulfotransferase, have been studied in any detail (Jain et al., 1989; Glendening and Poulton, 1990), and there have been some preliminary studies of the Met chain-extension system (Chapple et al., 1990). Much more is known about the synthesis of cyanogenic glucosides in sorghum and cassava, and this biosynthetic pathway apparently shares some intermediates with the glucosinolate pathway (Fig. 1B).

It has been shown clearly that the *N*-hydroxylation of precursor amino acids and the subsequent production of aldoximes are catalyzed by microsomal Cyt P-450-type enzymes in both sorghum (Halkier and Moller, 1991) and cassava (Koch et al., 1992). In the expectation that similar

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Abbreviations: ABT, 1-aminobenzotriazole; DHMet, dihomomethionine (2-amino-6-methylthiohexanoic acid); FAD, flavin adenine dinucleotide; FMN, flavin adenine mononucleotide; HPhe, homophenylalanine (2-amino-4-phenylbutyric acid).

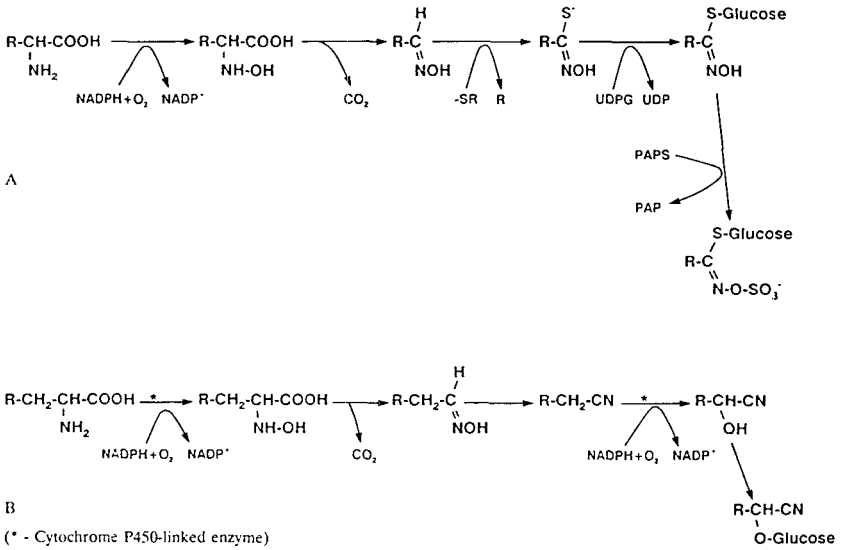

Figure 1. The proposed biosynthetic pathways leading to glucosinolates (A) and cyanogenic glucosides (B).

enzyme systems would function in glucosinolate-accumulating tissues of oilseed rape, we looked for appropriate enzyme activities in microsomal preparations from young leaves. Young, expanding, green leaves had earlier been shown to rapidly accumulate all classes of glucosinolates (Porter et al., 1991). Enzymes that metabolize both DHMet and HPhe were detected in rape leaf microsomes, as reported elsewhere (Dawson et al., 1993). We describe here the characteristics of these enzyme systems and provide evidence that at least two distinct enzymes are involved with some characteristics that suggest they may be flavin-containing monooxygenases.

MATERIALS AND METHODS

Chemicals

D,L-[1-^{14}C]HPhe and D,L-[1-^{14}C]DHMet were synthesized as described elsewhere (Dawson et al., 1993). Bovine liver catalase and horseradish superoxide dismutase were obtained from Sigma. All other chemicals were analytical grade.

Plant Material

Oilseed rape (*Brassica napus* L., cv Bienvenu) seeds were obtained from Rothamsted farm stocks. Plants were grown in compost in a controlled environment room under the following conditions: 12 h light (a combination of tungsten and fluorescent lamps, 350 μE m^{-2} s^{-1} average PAR), 17/14°C day/night temperature, 80/90% day/night RH.

Preparation of Microsomes

All operations were carried out on ice in a cold room (4°C), and the pestle and mortar and all buffer solutions were prechilled. A mixture of leaves 5 and 6 from 48-d-old plants was used in all experiments, because these leaves gave the highest activity. Twenty grams (fresh weight) of leaves were chopped and then homogenized in a pestle and mortar with 10 g of acid-washed sand and 100 mL of homogenization buffer (0.25 M Suc, 50 mM Na bisulfite, 10 mM ascorbic acid, 0.1 M KH_2PO_4, adjusted to pH 7.0 with KOH). The resulting homogenate was slurried with 10 g of polyvinylpolypyrrolidone and 2 g of Amberlite XAD-4 for 2 min and then filtered through four layers of muslin. The residue was washed with an additional 50 mL of buffer and was filtered again through muslin. The filtrates were combined and centrifuged at 27,000*g* for 20 min. The supernatant was centrifuged at 100,000*g* for 1 h, and the resulting pellet was resuspended in 2× 500 μL of resuspension buffer (20% [v/v] glycerol, 0.1 M KH_2PO_4, adjusted to pH 7.5 with KOH). The suspension was transferred to Eppendorf tubes and centrifuged for 10 min in a Biofuge. The pooled supernatants were made up to 5 mL with resuspension buffer, and the resulting solution comprised the crude microsome preparation used in all assays.

Enzyme Assays

Metabolism of HPhe or DHMet by the microsome preparations was monitored by following the release of $^{14}CO_2$ from substrates with label in the carboxyl group. Both substrates were obtained at a specific activity of 800 MBq mmol^{-1} and were diluted to 222 KBq mL^{-1}. Glass vials (5 × 2 cm) were used for the assays, with a smaller glass vial placed within containing 1 mL of 1 M KOH plus a wick of Whatman No. 1 filter paper. Assays consisted of 200 μL of microsomes in a final total volume of 500 μL, and all effectors were dissolved in resuspension buffer unless otherwise stated. Reactions were started by the addition of 25 μL of substrate (containing 6.8 nmol), and the vials were capped with a Subaseal before incubation at 30°C for 30 min. The reaction was stopped by injecting 30 μL of 40% (v/v) HCl into the assay mix. After overnight incubation at 30°C (to allow $^{14}CO_2$ released from the acidified assay mix to be trapped in the KOH solution), a 500-μL aliquot of KOH solution was added to 5 mL of Cocktail T (BDH Chemicals). Radioactivity was determined using a Packard 2500T scintillation counter. All assays were run in triplicate and compared to control assays containing only microsomes, substrate, and buffer.

For some experiments, an NADPH-generating system was added to the reaction mixture. This contained Glc-6-P dehydrogenase (2.5 units), Glc-6-P, and NADPH. The latter two components were dissolved in resuspension buffer.

Cofactors, inorganic salts, Cyt P-450, heme inhibitors, and amino acid-modifying reagents were dissolved in resuspension buffer and preincubated with the microsome preparations for 10 min before the addition of substrate. ABT was dissolved in DMSO before addition, and a DMSO control was run with these assays. Amino acids and methimazole were injected simultaneously with the substrate. To test the effect of carbon monoxide, the gas (99.5% by volume) was bubbled through an aliquot of microsomes for 2 min. As a control, a similar aliquot was treated identically using O_2-free N.

RESULTS

We previously showed that the NADPH-dependent decarboxylation of HPhe by rape leaf microsomes produces the appropriate aldoxime (Dawson et al., 1993) that is predicted if the enzyme(s) is (are) part of the glucosinolate biosynthetic pathway. (The equivalent oxime from DHMet is unstable in the enzyme reaction mixture, and therefore, direct demonstration of its formation is not possible.) The release of $^{14}CO_2$ from either HPhe or DHMet labeled in the 1 position is absolutely dependent on the presence of NADPH, and no activity was detected with any other cofactor (Table I), although some slight stimulation by flavins of the NADPH-dependent activity was apparent. This FAD plus FMN stimulation was more marked in some early experiments, before the extraction procedure was optimized (data not shown). Addition of an NADPH-generating system to the assay markedly inhibited both activities, but this appears to be a consequence of the high ammonium sulfate content of the Glc-6-P dehydrogenase used.

The equivalent enzyme system of the cyanogenic glucoside pathway in sorghum contains at least one Cyt P-450; therefore, a variety of classic Cyt P-450 inhibitors were tested. As shown in Table II, although ABT inhibited both activities by 40 to 45%, this effect could be almost wholly accounted for by the inhibition due to the solvent used, DMSO. Likewise, the slight inhibition caused by bubbling reaction mixtures with CO was identical with that found after bubbling with N_2. Addition of Cyt *c* to the reaction caused no more than 20% inhibition, and neither ferricyanide nor sodium azide affected either activity. From this it would appear that neither a Cyt P-450 nor a heme group is associated with the aldoxime-forming enzyme system(s). DTT, normally required to protect Cyt P-450 activity in microsomes (Hallahan et al., 1992), was inhibitory if present in either homogenization or resuspension buffers (Table III). Neither superoxide dismutase nor catalase had any effect on the enzyme activities when added (50 units) to the reaction mixture (data not shown).

Nonspecific amino acid decarboxylase activity could not be detected in the microsome preparations with the use of a variety of ^{14}C-labeled amino acids (data not shown). Release of $^{14}CO_2$ from the two glucosinolate precursor amino acids was not significantly inhibited by a 40-fold excess of other amino acids (Table IV), suggesting a high degree of substrate specificity for the enzyme(s). In particular, Phe, Tyr, and Trp did not inhibit the HPhe activity, although Met inhibited DHMet activity by up to 20%. Neither of the substrates (added as the unlabeled compound in 40-fold excess) inhibited $^{14}CO_2$ release from the other substrate, suggesting that

Table I. *Cofactor requirements for $^{14}CO_2$ release from amino acids by rape leaf microsomes*

D,L-[1-^{14}C]HPhe or D,L-[1-^{14}C]DHMet was incubated with rape leaf microsomes as described in "Materials and Methods," and the $^{14}CO_2$ released during incubation at 30°C for 30 min was collected and counted. Activity is expressed as percentage of that found with NADPH; 100% activity is equivalent to between 250 and 370 nmol of CO_2 released h^{-1} g^{-1} fresh weight.

Cofactor	Concentration	HPhe Activity	DHMet Activity
	μM	%	%
None	—	0	0
ATP	20	0	0
Mg·ATP	20	0	0
CoA	20	0	0
Pyridoxal phosphate	20	0	0
NADH	500	0	0
NADPH	500	100	100
NADPH + FAD + FMN	500 + 10 + 10	133	103

Table II. *The effect of Cyt P-450 and heme inhibitors on the release of $^{14}CO_2$ from HPhe and DHMet*

CO and N_2 were bubbled through the resuspended microsomes for 2 min before the assay. Other compounds were added to the standard assay mixture to the final concentration shown, before addition of substrate, and conditions were otherwise as for Table I.

Compound/Treatment	Concentration	HPhe Activity	DHMet Activity
		% control activity	
Cyt c	0.2 μM	85	79
1-ABT (in DMSO)	4 mM	59	55
DMSO		69	67
CO		94	85
N_2		94	84
NaFeCN	0.2 mM	113	105
Na azide	6 mM	91	99

Table III. *Effect of DTT in extraction and resuspension buffers on $^{14}CO_2$ release from amino acids*

DTT (1 mM) was included in (+) or omitted from (−) the initial homogenization (H) buffer and/or the microsome resuspension (R) buffer. Enzyme activity was then assayed using the standard conditions, with 100% activity as described in Table I.

Substrate	Treatment			
	H−/R−	H+/R+	H+/R−	H−/R+
HPhe	100	49	59	71
DHMet	100	59	88	78

two separate enzymes are present in the microsomes, each specific for one of the chain-extended amino acids. Methimazole, reported to be a substrate for flavin-containing monooxygenases from mammalian tissues (Coecke et al., 1992), weakly stimulated both activities, and imidazole stimulated the DHMet activity but not that with HPhe (Table IV). Other amines (and hydroxylamine) had little or no effect on either activity (data not shown).

Optimum assay pH for both activities was 7.5 (Fig. 2), but the pH curves for the two activities are somewhat different. For the HPhe activity, greater than 80% of the maximum activity occurs from pH 6.5 to 8.0, whereas the DHMet activity decreases sharply above or below pH 7.5.

A variety of other potential inhibitors were investigated to determine the nature of the enzyme activities being studied (Table V). Chelating agents, in general, were not inhibitory, or only weakly so, although 200 μM NaFeEDTA caused a 60% inhibition of both activities. Copper salts, however, were very potent inhibitors, although no other metal ions had any effect at concentrations up to 200 μM. Ammonium sulfate added at the same concentration as found in the NADPH-generating system was inhibitory, although low concentrations (<1 mM) were not. Neither *N*-ethyl maleimide nor PMSF had any effect, but diethyl pyrocarbonate was a very potent inhibitor, suggesting the presence of a His or Tyr residue necessary for catalytic activity on at least one of the enzymes of the system.

Further purification of the enzymes, and analysis of the number of enzymes involved, requires the removal of the proteins from the microsomal membranes. However, CHAPS or Triton X-100 treatment of the microsomes greatly reduced the activity of both enzyme systems, and 1% detergent totally abolished activity (Table VI). To determine the apparent K_m (K_m') for HPhe, a more concentrated stock solution of the substrate was used (666 KBq mL^{-1}), and assays were performed at a variety of concentrations and times (10–50 min).

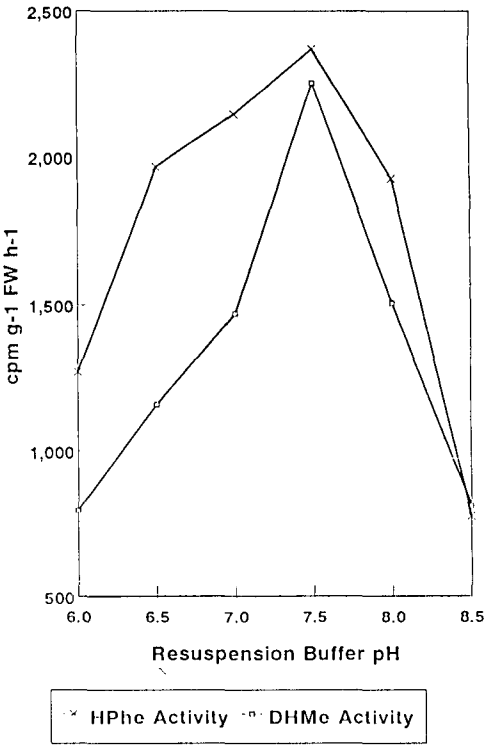

Figure 2. The effect of pH on the release of $^{14}CO_2$ from DHMet and HPhe by rape leaf microsomes. Microsomes were resuspended in standard medium adjusted to the pH shown, and both enzyme activities were determined. FW, Fresh weight.

Table IV. *Effect of amino acids and other compounds on $^{14}CO_2$ release from HPhe and DHMet*

Assays were performed with 13.6 μM ^{14}C-labeled substrate in the presence of amino acids and the other compounds listed at the concentrations shown. Unlabeled HPhe and DHMet were added to assays containing the opposite labeled substrate. Activities are quoted as percentage of control activity (no additions), as described for Table I. nd, Not determined.

Addition	Concentration	HPhe Activity	DHMet Activity
	μM	%	%
None		100	100
L-Phe	500	95	87
L-Trp	500	100	100
L-Tyr	500	94	97
L-Met	500	91	80
L-Lys	500	nd	100
L-HPhe	500		104
L-DHMet	500	119	
Methimazole	100	121	102
Methimazole	500	120	115
Imidazole	200	100	140
Imidazole	500	100	249

Table V. *Inhibition of $^{14}CO_2$ release from HPhe and DHMet by inorganic salts, chelating agents, and amino acid modification reagents*

Compounds were preincubated with microsomes in the assay mix at the final concentrations shown, before the addition of substrates. Activities were measured and expressed as for Table I.

Addition	Concentration	HPhe Activity	DHMet Activity
	μM	%	%
None		100	100
$CuCl_2$	20	63	76
$CuCl_2$	100	5	1
$CuSO_4$	20	64	83
$CuSO_4$	100	6	2
CuCl	100	0	0
$(NH_4)_2SO_4$	130 mM	26	27
Other salts[a]	200	100	100
NaEDTA	200	80	90
NaFeEDTA	200	39	40
EGTA	200	90	86
N-ethyl maleimide	200	97	89
PMSF	200	100	100
Diethyl procarbonate	20	1	13

[a] "Other salts" were MgCl, $MgSO_4$, $FeCl_2$, $FeCl_3$, $Fe[NO_3]_3$, Na_2SO_4, K_2SO_4, Na_2MoO_4, and Na_2WO_4.

Table VI. *The effect of detergents on $^{14}CO_2$ release from HPhe and DHMet by rape leaf microsomes*

Triton X-100 or CHAPS were included in the microsome resuspension buffer at the final concentration shown. Activity was determined and expressed as for Table I.

Addition to Buffer	Concentration	HPhe Activity	DHMet Activity
	% (w/v)	%	%
None		100	100
CHAPS	0.1	57	68
CHAPS	1.0	0	0
Triton	0.1	43	53
Triton	1.0	0	0

At all concentrations of substrate, activity was proportional to time for at least 30 min. Using the direct linear plot (Eisenthal and Cornish-Bowden, 1974), we obtained estimated K_m' of 50 μM for D,L-HPhe. If the L-isomer is the true substrate, this would be equivalent to a K_m' (L-HPhe) of 25 μM. Preliminary experiments with the DHMet enzyme suggest that it has a somewhat higher K_m' of about 100 μM.

DISCUSSION

Microsomes prepared from young expanding leaves of oilseed rape contain enzyme systems that catalyze the NADPH-dependent oxidative decarboxylation of HPhe and DHMet, with the production of the aldoxime in the former case. The high specificity of these enzyme systems for the chain-extended amino acids suggests that they are involved in the biosynthesis of 2-phenylethyl- and 3-butenyl-glucosinolates, both of which accumulate in developing rape leaves (Porter et al., 1991). Similar NADPH-dependent aldoxime formation from amino acids has been found in microsomes from sorghum and cassava (Halkier and Moller, 1991; Koch et al., 1992), but in contrast to those reports, we cannot detect the involvement of any Cyt P-450 in the rape leaf activities. From our studies with potential inhibitors, there is, likewise, no indication of a heme-containing electron transfer component of the system, and the failure of *N*-ethyl maleimide to inhibit activity (plus the negative effects of DTT) would seem to rule out the presence of an essential sulfydryl group on any of the proteins involved.

The involvement of aldoximes in glucosinolate biosynthesis has been questioned (Halkier et al., 1991), largely on the basis of comparisons with the cyanogenic glucoside pathway in sorghum. In our microsomal system, the only detectable product from HPhe is the relevant aldoxime (Dawson et al., 1993), no trace of nitro compounds being found, and in view of the very different nature of the rape leaf *N*-hydroxylation enzymes from their counterparts in sorghum, it would be unwise to assume that the metabolic pathways are similar in other respects. The only data implicating the involvement of intermediates other than aldoximes in glucosinolate biosynthesis came from rather crude feeding studies with the noncrucifer *Tropaeolum majus* (Matsuo et al., 1972). In that study, nonenzymic interconversions between possible intermediates, particularly during extraction, were not taken into consideration.

The precise nature of the oxygenase system(s) in rape leaves has to be confirmed, but similar *N*-hydroxylation reactions are characteristic of flavin-containing monooxygenases found in mammalian liver, lung, and other tissues (for a review, see Ziegler, 1988). Although the activity in microsomes prepared from rape leaves under optimal conditions is only weakly stimulated by flavins, some early experiments gave a 2- to 3-fold stimulation by FAD plus FMN. The isolation conditions used at that time could have caused a loss of enzyme-bound flavin, which our improved extraction methods avoid. We are not aware of any reports of *N*-hydroxylation or aldoxime formation from amino acids by flavin-containing monooxygenases, but the rabbit lung enzyme catalyzes oxime formation from primary amines via the *N*-hydroxylamine (Poulsen et al., 1986). Methimazole is commonly used as a substrate for such monooxygenases (Coecke et al., 1992), but we could not detect any activity with this compound (measured as inhibition of CO_2 release from the amino acid substrates). This might simply reflect the apparently tight substrate specificity of the rape leaf enzymes, in contrast to the broad substrate specificity of the animal enzymes. Further work is needed to determine whether the rape leaf enzymes are related to the flavin monooxygenases. We have not been able to detect the involvement of activated oxygen species in the reaction(s); neither catalase nor superoxide dismutase have any influence on the activities.

It is likely that the overall reaction we are measuring, aldoxime formation with the release of CO_2, is catalyzed by more than one enzyme. The ability of both *N*-hydroxy and oximino acid derivatives of HPhe (and the aldoxime) to inhibit CO_2 release from HPhe, as demonstrated earlier (Dawson et al., 1993), indicates that they may be intermediates in the reaction, to some extent exchangeable with the solution rather than tightly bound to an enzyme. *N*-Hydroxyphenylalanine has been shown to be a better precursor of benzylglucosinolate than Phe in whole plant-feeding studies, and a cell-free system was obtained that converted *N*-hydroxyphenylalanine to phenylacetaldehyde aldoxime without a requirement for NAD(P)H or other cofactors (Kindl and Underhill, 1968). This would support the idea that it is the *N*-hydroxylation step in our system that is NADPH dependent and that a second enzyme is present that catalyzes aldoxime formation. The idea of a complex is supported by the inactivation of CO_2 release by detergents, suggesting that, for the overall reaction to proceed, two (or more) enzymes need to be in close proximity in an intact membrane (although direct inhibition by the detergents cannot be ruled out yet). Inhibition of the reaction by the product, the aldoxime, could simply be a question of mass action, but it is also possible that the aldoxime acts as a feedback inhibitor of the first enzyme. This would restrict flux into the pathway under conditions in which insufficient S donors were available for further metabolism of the aldoxime to the thiohydroximate and, therefore, glucosinolate. The whole question of the regulation of glucosinolate metabolism has yet to be addressed at the enzyme level. Further work is needed to dissect the enzymes of the aldoxime-forming system from the microsomal membranes and to look for the partial activities.

Despite the close similarity of the proposed metabolic pathways and reaction intermediates of glucosinolate and

cyanogenic glucoside biosynthesis, there would appear to be no common enzyme system responsible for amino acid *N*-hydroxylation in plants. Indeed, a third and completely different aldoxime-forming enzyme system has been found in Chinese cabbage. This is a membrane-bound peroxidase that converts Trp to indole-3-acetaldaldoxime (Ludwig-Muller and Hilgenberg, 1988; Ludwig-Muller et al., 1990). Such activity is not restricted to glucosinolate-accumulating plants, and indole-3-acetaldaldoxime has been proposed as a precursor of IAA (Helmlinger et al., 1987). The apparently common features of the glucosinolate and cyanogenic glucoside systems would seem to be an example of convergent evolution, the two systems having developed independently in different families of flowering plants. It would be intriguing to uncover the mechanism(s) for *N*-hydroxylation and aldoxime formation in *Carica papaya*, which uniquely appears to contain both classes of secondary compound (Spencer and Seigler, 1984). Quite possibly the enzyme system(s) might be a better taxonomic marker than simply the presence of either glucosinolates or cyanogenic glucosides.

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