Plant Physiol. (1997) 114: 1283–1291

Involvement of Cytochrome P450 in Glucosinolate Biosynthesis in White Mustard[1]

A Biochemical Anomaly

Richard N. Bennett, Guy Kiddle, and Roger M. Wallsgrove*

Biochemistry and Physiology Department, IACR-Rothamsted, Harpenden AL5 2JQ, United Kingdom

One of the first steps in glucosinolate biosynthesis is the conversion of amino acids to their aldoximes. The biochemistry of this process is controversial, and several very different enzyme systems have been described. The major glucosinolate in white mustard (*Sinapis alba*) is sinalbin, which is derived from tyrosine via its aldoxime, and this conversion is catalyzed by a cytochrome P450 (Cyt P450) monooxygenase. Phenylethyl- and alkenylglucosinolates are also present in white mustard leaves, as are the enzymes catalyzing the relevant aldoxime formation from homophenylalanine and methionine homologs, respectively. These enzymes are similar to those found in *Brassica* sp. and are distinct from the tyrosine-dependent enzyme in that they contain no heme and are unaffected by Cyt P450 inhibitors. They are instead inhibited by the flavoprotein inhibitor diphenylene iodonium and by Cu^{2+}. In both white mustard and oilseed rape (*Brassica napus*) methyl jasmonate specifically stimulates indolylglucosinolate biosynthesis and yet has no effect on sinalbin accumulation in either cotyledons or leaves of white mustard. White mustard appears to be unique among crucifers in having a Cyt P450 aldoxime-forming enzyme for biosynthesis of one glucosinolate, although it also contains all of the non-Cyt P450 enzyme systems found in other members of the family. Sinalbin biosynthesis in white mustard is therefore an inappropriate model system for the synthesis of other glucosinolates in crucifers, including canola and oilseed rape.

Considerable efforts have been made to breed glucosinolates out of the seeds of commercial varieties of canola (*Brassica campestris*) and oilseed rape (*Brassica napus*), because the presence of these compounds reduces the nutritional value of rapeseed meal. However, these same secondary metabolites are present in cruciferous vegetables eaten by humans. Their breakdown products (isothiocyanates and others) are the flavor components of mustards, radishes, broccoli, and others. The presence of moderate quantities of such compounds in foodstuffs has been found to have significant beneficial effects, such as the stimulation of defensive responses that protect against carcinogens (Jongen, 1996; Rhodes, 1996). The successful reduction in seed glucosinolates in canola and rape is detrimental to the survival of the emerging seedlings, because low glucosinolate content of cotyledons encourages feeding by slugs and other nonspecialist herbivores (Glen et al., 1990). In the plant glucosinolates have a major role in defense against herbivores and microorganisms, although they also act as attractants for some specialist insect pests (Bennett and Wallsgrove, 1995; Bartlett, 1996). Understanding the complex biology of such interactions, along with the development of directed breeding programs and genetic manipulation, may allow the development of more resistant crops that retain optimum end-user properties.

Aldoximes, presumed to be derived from *N*-hydroxyamino acids, are early intermediates in glucosinolate biosynthesis (Underhill, 1980). They are also intermediates in cyanogenic glucoside biosynthesis, and this commonality led to the assumption that the biochemistry of the two pathways would likewise be similar (Poulton and Møller, 1993). In cyanogenic plants *N*-hydroxylation of amino acids is catalyzed by Cyt P450 (heme-thiolate protein) MOs (Halkier and Møller, 1991), and similar Cyt P450 enzymes active with Tyr or Phe have been identified in white mustard (*Sinapis alba*) (Du et al., 1995; Bennett et al., 1996), *Tropaeolum majus*, and *Carica papaya* (Bennett et al., 1996, 1997), all of which contain glucosinolates. However, no such Cyt P450 MOs have ever been demonstrated in extracts from *Brassica* sp.; instead, there is evidence for two other aldoxime-forming enzyme systems.

Plasma membrane peroxidases catalyzing the formation of indole acetaldoxime from Trp have been found in many plant species, including those that do not make glucosinolates (Ludwig-Muller and Hilgenberg, 1988); in *Brassica* sp. the activities of these enzymes are high and their developmental regulation closely follows the pattern of indole glucosinolate accumulation (Bennett et al., 1995b). NADPH-dependent MOs catalyzing aldoxime formation from chain-extended amino acids (precursors of alkenylglucosinolates and aromatic glucosinolates in *Brassica* sp.) have also been identified and characterized. Two enzymes appear to be present in all *Brassica* sp. examined, one specific for HPhe and one specific for Met homologs (Bennett et al., 1993,

[1] This research was supported by grants from the Ministry of Agriculture, Fisheries and Food, UK, and funding of IACR by the Biotechnology and Biological Sciences Research Council, UK.

* Corresponding author; e-mail roger.wallsgrove@bbsrc.ac.uk; fax 44-582-760-981.

Abbreviations: DHMet, dihomomethionine; DPI, diphenylene iodonium; HPhe, homophenylalanine; MJ, methyl jasmonate; MO, monooxygenase; pHBG, *p*-hydroxybenzylglucosinolate (sinalbin); SA, salicylic acid.

1995a, 1995b, 1996). In a few *Brassica* sp. an L-Phe MO was also detected. These membrane-bound enzymes are insensitive to CO and other inhibitors of Cyt P450s and heme-containing enzymes and are strongly inhibited by the flavoprotein inhibitor DPI (O'Donnell et al., 1993) and Cu^{2+} ions (Bennett et al., 1993, 1995a, 1995b, 1996). Figure 1 shows the relationship between precursor amino acids, aldoximes, and the glucosinolates derived from them.

A recent study of Cyt P450 MO activity in etiolated white mustard seedlings was claimed as experimental proof of close similarity between cyanogenic glucoside and aromatic glucosinolate biosynthesis (Du et al., 1995). The authors cast doubt on the involvement of other aldoxime-forming activities in glucosinolate biosynthesis. However, it remains an open question whether the Cyt P450 MO activity found in these etiolated seedlings is related to glucosinolate biosynthesis, because previous work indicated that cotyledons do not actively synthesize aromatic or aliphatic/alkenylglucosinolates (Glen et al., 1990; Bodnaryk, 1991), and glucosinolate biosynthesis in leaf tissues is strongly light dependent (Milford and Evans, 1991). The work on white mustard also showed that this Cyt P450 MO activity was stimulated by jasmonate treatment of the seedlings (Du et al., 1995), although others have found that jasmonates only stimulate indolylglucosinolate formation (Bodnaryk, 1992, 1994; Doughty et al., 1995). Specifically, jasmonate treatment of white mustard cotyledons was reported to have no effect on sinalbin (pHBG) (Bodnaryk, 1992, 1994). In addition, the reported enzyme activity (Du et al., 1995) was very low, i.e. approximately 1000-fold less than aldoxime-forming activities detected in leaf tissue of white mustard or *Brassica* sp. (Bennett et al., 1996).

We have therefore re-examined glucosinolate biosynthesis in white mustard with respect to the presence and nature of aldoxime-forming enzymes and the effects of elicitors and light. In this report we demonstrate that white mustard leaves contain MO activities similar to those previously reported in oilseed rape, which share no characteristics with Cyt P450 MOs, and contain a Tyr-dependent Cyt P450 MO not present in other crucifers. There is no evidence that jasmonates (or salicylates) induce synthesis of pHBG in white mustard, suggesting that

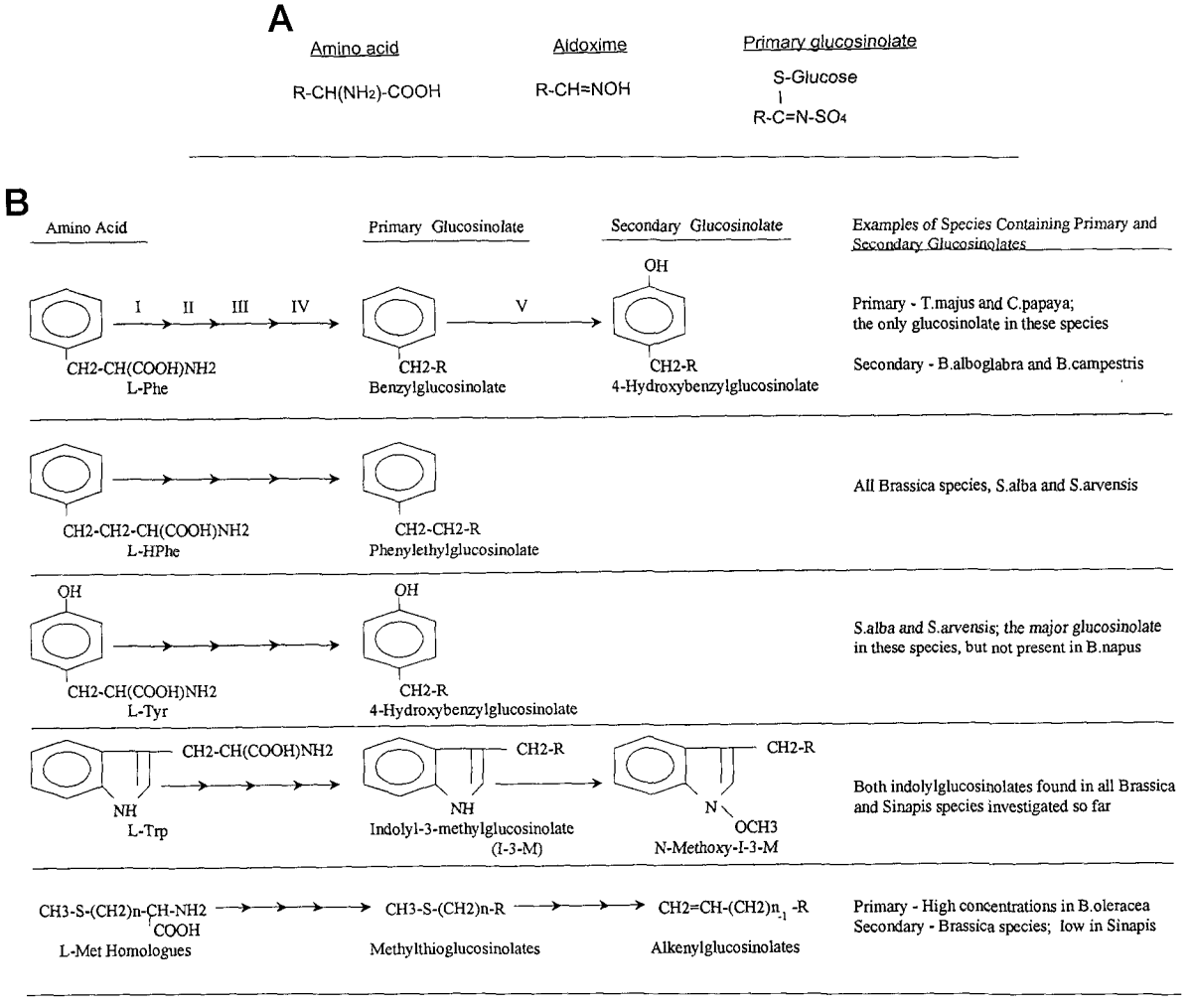

Figure 1. A, Conversion of amino acids to aldoximes with the loss of CO_2. R, Amino acid side chain. B, Relationship between amino acids and glucosinolates. The enzymic steps involved are: I, aldoxime formation; II, thiohydroximate formation; III, glucosylation; IV, sulfation; V, side-chain modification(s). R, Core glucosinolate structure, as shown in A.

the jasmonate-stimulated Cyt P450 MO reported in etiolated cotyledons (Du et al., 1995) may not be involved in glucosinolate biosynthesis. In evolutionary terms, white mustard appears to have acquired a new aldoxime-forming enzyme in addition to those derived from its common crucifer ancestry. Glucosinolate synthesis in crucifers and other plants probably evolved independently and in crucifers apparently did not derive from ancestral cyanogenesis.

MATERIALS AND METHODS

Synthesis of 1-^{14}C-amino acids and DPI was as reported previously (Dawson et al., 1993; Bennett et al., 1996). CO (99.5% by volume) and O_2-free N_2 were obtained from BDH (Poole, UK) and BOC (Guildford, UK), respectively. All other chemicals were from Sigma.

Seeds of white mustard (*Sinapis alba*) were obtained from E.W. King (Suffolk Herbs, UK) and those of oilseed rape (*Brassica napus* cv Bienvenu) were obtained from Rothamsted stocks. Light-grown seedlings and plants were grown in a greenhouse (12-h light/12-h dark combination of tungsten and fluorescent lamps giving 350 μmol m^{-2} s^{-1} average PAR, 17/14°C day/night temperature). The seeds used for the production of the etiolated seedlings were sterilized with dilute hypochlorite solution and sown on filter paper. The seedlings were grown in the dark for 7 d, treated with elicitors, and then returned to the dark for a further 3 d.

Glucosinolate Extraction and Analysis

Glucosinolates were extracted and analyzed by HPLC of desulfoglucosinolates, as previously reported (Porter et al., 1991; Bennett et al., 1995b). Peak identification was by reference to the retention time and UV/visible spectrum (from the diode-array detector on the HPLC) of standards, which had been independently characterized by GC-MS. A minimum of three independent experiments were conducted to determine the content of glucosinolates in seeds, etiolated and light-grown seedlings, and plants after the various treatments.

Treatment of Plants with Elicitors

Seven-day-old seedlings (etiolated and light-grown) and 30-d-old mature plants of white mustard and oilseed rape were used. MJ was applied as a 100 μM spray (in 50% [v/v] ethanol) to the foliar parts of the plants; 50% (v/v) ethanol-treated and water-treated controls were used in all experiments. SA (2.5 mM) was applied as a soil drench; control plants were treated with distilled water. Samples (cotyledons from seedlings; leaf 6 from mature plants) were collected for glucosinolate analysis 3 d after treatment.

Preparation of Microsomes

Microsomes were prepared from young, expanding green leaves as previously described (Bennett et al., 1993, 1995a, 1996). Two separate microsomal preparations were made from white mustard tissues, using conditions optimized for either the Cyt P450s or for flavoproteins (Bennett et al., 1996).

MO Assays

The assays were performed as previously described (Bennett et al., 1996). Activity was determined as the NADPH-dependent release of $^{14}CO_2$ from 1-^{14}C-labeled amino acids. NADPH-independent amino acid decarboxylase activity was minimal, as was found previously (Bennett et al., 1996). Cyt P450 inhibitors were added in DMSO to give a final concentration of 2.5 mM. DPI was added in DMSO to give a final concentration of 1 mM. All additions in DMSO were compared with DMSO controls. All of the other inhibitors were added in the resuspension buffer to give the final concentrations required: NaCN and NaN_3, 5 mM; $CuCl_2$, 250 μM; and Cyt *c*, 2 mM. All inhibitors were preincubated with microsomes in the assay system for 10 min at 30°C before the amino acid substrate was added. CO and N_2 were bubbled through 1 mL of microsomes for 2 min, and then 200-μL aliquots were removed and added to the standard assays (Bennett et al., 1996). All inhibitors were tested in triplicate in a minimum of six independent experiments for each species.

Extraction and Assay of Microsomal Peroxidases

Peroxidase activity in the microsomal fractions prepared as described above was measured spectrophotometrically by following the formation of tetraguaiacol from guaiacol at 470 nm. The reaction was initiated by the addition of 50 μL of extract to 950 μL of assay buffer (50 μM H_2O_2 and 50 μM guaiacol in 200 mM potassium phosphate, pH 5.8). The inhibitors described above were tested at the same final concentrations. All peroxidase assays were done in triplicate in at least six independent experiments for each species.

Extraction and Assay of Glutathione Reductase

Glutathione reductase was extracted from young, green expanding leaves of white mustard and oilseed rape and partially purified using the procedures reported by Carlberg and Mannervik (1985), with the following modifications. The leaves were homogenized at 4°C with 6.5 mL g^{-1} fresh weight of extraction buffer (1 mM EDTA, 0.1 mM DTT, 6 μM FAD, 50 μM PMSF, and 0.25 M Suc in 0.1 M potassium phosphate, pH 7.0). The homogenate was immediately mixed with PVP and hydrophobic resin beads (Amberlite XAD-4, Sigma) (0.45 and 0.05 g g^{-1} fresh weight, respectively), and filtered through four layers of muslin. The filtrate was centrifuged (7000*g*, 60 min, 4°C), the supernatant was removed, and the pH was adjusted to 5.3 with phosphoric acid. The pH-adjusted supernatant was centrifuged (15,000*g*, 40 min, 4°C) and the final supernatant was adjusted to pH 7.5 with 1 M KOH.

Glutathione reductase activity in the extracts was measured as described by Fitzgerald et al. (1991). The reaction was initiated by the addition of 50 μL of extract to 950 μL

of assay buffer (6.3 mM EDTA in 0.125 M potassium phosphate, pH 7.4, 0.3 mM oxidized glutathione, and 0.3 mM NADPH). All assays were done in triplicate in a minimum of three independent experiments.

RESULTS

Glucosinolates in Cotyledons and Leaves of White Mustard and Oilseed Rape

The only glucosinolate detected in cotyledons of both etiolated and light-grown white mustard was pHBG (Table I). Cotyledons of 10-d-old etiolated seedlings had a similar but a little lower glucosinolate content compared with mature seeds, whereas in light-grown plants the glucosinolate content was greatly reduced, suggesting that under these conditions glucosinolates were being metabolized (dry weights of seeds and cotyledons were similar across all treatments). Neither of the elicitor treatments, MJ or SA, had any major effect on the glucosinolate content of white mustard seedlings, although in both etiolated and green cotyledons MJ treatment caused a slight (15%) decrease. Oilseed rape seeds and seedlings contained no pHBG but did contain alkenyl-, indolyl-, and phenylethylglucosinolates. The indolylglucosinolate content of these seedlings was a little higher than in dry seed, whereas the contents of the other glucosinolates were reduced compared with the seed. As found with white mustard cotyledons, light reduced the glucosinolate content of oilseed rape seedlings, affecting all three classes of glucosinolate (Table I). Elicitor treatments produced a significant change in glucosinolate content and profile in oilseed rape in both light- and dark-grown seedlings. SA specifically increased phenylethylglucosinolate and MJ specifically increased the indolylglucosinolates. Neither treatment had any effect on the other classes of glucosinolates.

Essentially the same response was found in true leaves of white mustard and oilseed rape (Table II). In contrast to cotyledons, true leaves of white mustard had significant alkenyl-, phenylethyl-, and indolylglucosinolates, although pHBG still made up more than 80% of the total. There was no change in the content of this latter compound in response to either elicitor treatment, whereas indolyl- and phenylethylglucosinolate contents increased markedly after treatment with MJ and SA, respectively, just as they did in oilseed rape true leaves (Table II; Kiddle et al., 1994; Doughty et al., 1995).

Aldoxime-Forming MO Activities in Leaves of White Mustard and Oilseed Rape

The identity and nature of NADPH-dependent, aldoxime-forming MO enzymes in white mustard leaves were investigated. Three NADPH-dependent activities were detected, utilizing Tyr, HPhe, and DHMet; the latter two activities were also found in oilseed rape leaf extracts. Optimum extraction of these two activities (from either white mustard or oilseed rape) required rather different conditions from those required to extract the Tyr-dependent enzyme. For the latter, DTT in the extraction and resuspension buffers markedly increased activity, whereas the other two activities were significantly inhibited, as reported previously (Bennett et al., 1993). No Tyr-dependent MO activity could be detected in oilseed rape leaf extracts using either extraction buffer.

A variety of potential inhibitors was tested on the three enzymes to determine the nature and characteristics of these MOs. Paclobutrazol, ancymidol, tetcyclasis, and 1-aminobenzotriazole are all potent inhibitors of Cyt P450s, and some of them were used to confirm the identity of a Tyr-dependent Cyt P450 MO in white mustard cotyledons (Du et al., 1995). We used slightly higher concentrations of these compounds (2.5 versus 1 mM) to ensure that the results were not significantly affected by any variation in sensitivity to these inhibitors between different Cyt P450 enzymes. The Tyr-dependent MO in white mustard leaves was inhibited 40 to 60% in the presence of any of the four inhibitors (Fig. 2). Bubbling the microsomes prior to assay with CO, a heme inhibitor classically used to define Cyt P450s because light reverses this inhibition (Du et al. 1995), also inhibited this reaction to a similar extent, whereas

Table I. *Glucosinolate content of seeds and cotyledons from etiolated and light-grown seedlings of white mustard and oilseed rape and effect of elicitors*

Data represent the means ± SE of duplicate determinations from each of three independent experiments. ALK, Total aliphatic glucosinolates/alkenyglucosinolates; PE, phenylethylglucosinolate; IND, total indolyglucosinolates.

Tissue	Treatment	White Mustard	Oilseed Rape		
		pHBG	ALK	PE	IND
		μmol g⁻¹ dry wt			
Seeds		339.5 ± 19.4	108.3 ± 10.2	94.3 ± 9.3	4.0 ± 0.3
Etiolated cotyledons	Control	221.9 ± 18.5	81.7 ± 8.6	4.3 ± 0.9	6.5 ± 0.6
	+ Ethanol	215.2 ± 18.1	80.7 ± 7.9	4.0 ± 0.9	6.3 ± 0.5
	+ 100 μM MJ	184.2 ± 17.7	80.4 ± 9.1	4.2 ± 0.5	10.6 ± 1.0[a]
	+ 2.5 mM SA	216.5 ± 18.9	80.8 ± 7.7	11.6 ± 2.1[a]	6.7 ± 1.0
Light-grown cotyledons	Control	85.6 ± 3.1	9.0 ± 1.2	0.5 ± 0.1	1.0 ± 0.1
	+ Ethanol	85.1 ± 4.0	9.6 ± 1.1	0.5 ± 0.1	1.1 ± 0.1
	+ 100 μM MJ	80.6 ± 3.4	8.8 ± 1.0	0.5 ± 0.03	6.9 ± 0.6[a]
	+ 2.5 mM SA	85.2 ± 4.1	8.9 ± 1.0	2.7 ± 0.4[a]	1.3 ± 0.3

[a] Values are significantly different from the control.

Table II. *Glucosinolate content in true leaves of white mustard and oilseed rape and the effect of elicitors*

Experimental details and glucosinolate abbreviations as for Table I.

Treatment	*S. alba*				*B. napus*		
	ALK	pHBG	PE	IND	ALK	PE	IND
				μmol g⁻¹ dry wt			
Control	27.1 ± 2.0	139.2 ± 17.1	0.8 ± 0.1	1.0 ± 0.1	71.2 ± 5.4	6.7 ± 1.0	4.5 ± 0.3
+ Ethanol	25.4 ± 1.9	143.8 ± 10.1	0.8 ± 0.03	1.3 ± 0.3	67.6 ± 4.8	6.6 ± 0.9	4.8 ± 0.5
+ 100 μM MJ	23.4 ± 2.0	139.2 ± 13.2	0.7 ± 0.05	17.5 ± 1.1[a]	67.4 ± 4.9	6.4 ± 1.1	76.3 ± 2.4[a]
+ 2.5 mM SA	24.9 ± 2.0	138.0 ± 14.1	4.4 ± 0.1[a]	1.1 ± 0.2	68.3 ± 7.3	12.6 ± 1.2[a]	4.4 ± 0.3

[a] Values are significantly different from the control.

treatment with N_2 had no effect. Light reversal of CO inhibition was not attempted, because the resuspended microsomes were opaque and dark green. CO likewise strongly inhibited microsomal peroxidases (heme-containing enzymes used as internal controls) from the same tissue and from extracts from oilseed rape leaves (Fig. 3). Peroxidase activities were not significantly affected by any of the Cyt P450 inhibitors, confirming that we were not observing nonspecific activities of these compounds on membrane proteins in general.

The HPhe- and DHMet-dependent MO activities were not significantly affected (<10% inhibition) by any of these inhibitors in either species, nor was glutathione reductase (a flavoprotein used as an internal control) from the same tissues (Figs. 2 and 3). Both of these MOs were, however, sensitive to DPI (a specific flavoprotein inhibitor, O'Donnell et al. [1993]) and Cu^{2+} ions, showing an 80 to 90% inhibition for each at the chosen concentration, but were insensitive to cyanide and azide, showing less than a 10% inhibition (Figs. 4 and 5). Glutathione reductase from both species was similarly sensitive to DPI and Cu^{2+} but not to cyanide or azide. The Tyr-dependent MO activity in white mustard leaves was strongly inhibited by cyanide and azide, as were the peroxidases in both species, and was also partially inhibited by Cu^{2+} ions. Cyt *c* was an inhibitor of all of the NAD(P)H-dependent enzymes, presumably because of competition for NAD(P)H from Cyt *c* reductase.

DISCUSSION

Elicitation of Glucosinolates in Cotyledons and Leaves

We found no evidence for an effect of MJ on pHBG content in cotyledons or true leaves of white mustard. Our data and those of other studies (Bodnaryk, 1992, 1994) clearly demonstrate that there is no stimulation of pHBG biosynthesis by this elicitor, but that MJ stimulates indolylglucosinolate formation in all of the crucifers examined (Bodnaryk, 1992, 1994; Doughty et al., 1995). Although SA markedly increased phenylethylglucosinolate synthesis in both oilseed rape and white mustard, this elicitor did not affect pHBG. Therefore, neither MJ nor SA treatment would be expected to induce or stimulate any of the enzymes involved in pHBG biosynthesis. Cotyledons, either etiolated or green, do not accumulate pHBG or other glucosinolates (except the indolyl compounds); rather, there is a light-stimulated degradation of these compounds. We have also observed that HPhe- and DHMet-dependent MO activities are essentially undetectable in unelicited green cotyledons of oilseed rape, although HPhe-dependent MO activity can be detected in

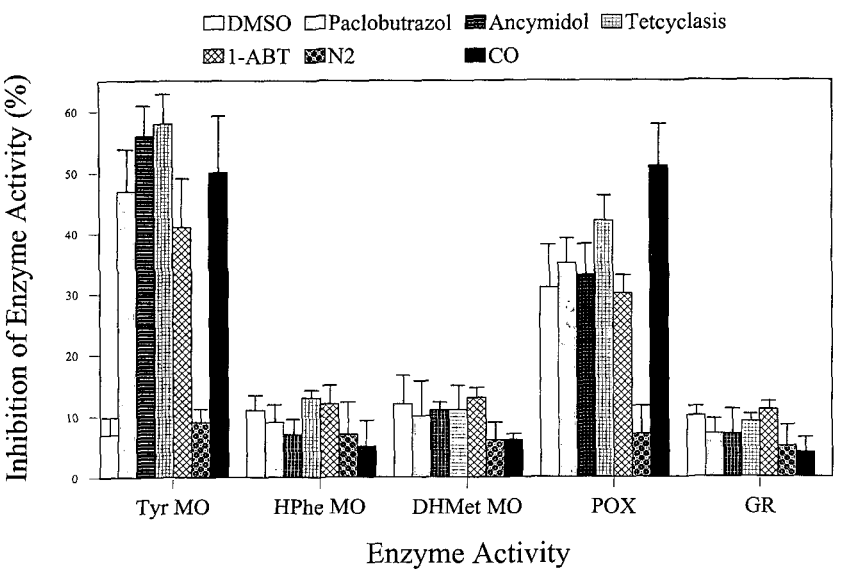

Figure 2. Effects of Cyt P450 inhibitors on enzymes from white mustard leaves. All inhibitors were dissolved in DMSO prior to addition to the assay mixture; CO and N_2 were bubbled through microsome suspensions as detailed in "Materials and Methods." POX, Microsomal peroxidase. For each enzyme 100% activity (mean of six independent experiments) was: Tyr MO, 0.575; HPhe MO, 0.458; DHMet MO, 0.895 (all μmol CO_2 mg^{-1} microsomal protein h^{-1}); POX, 72 nmol tetraguaiacol g^{-1} fresh weight h^{-1}; and glutathione reductase (GR), 187 nmol NADPH oxidized g^{-1} fresh weight h^{-1}. 1-ABT, 1-Aminobenzotriazole.

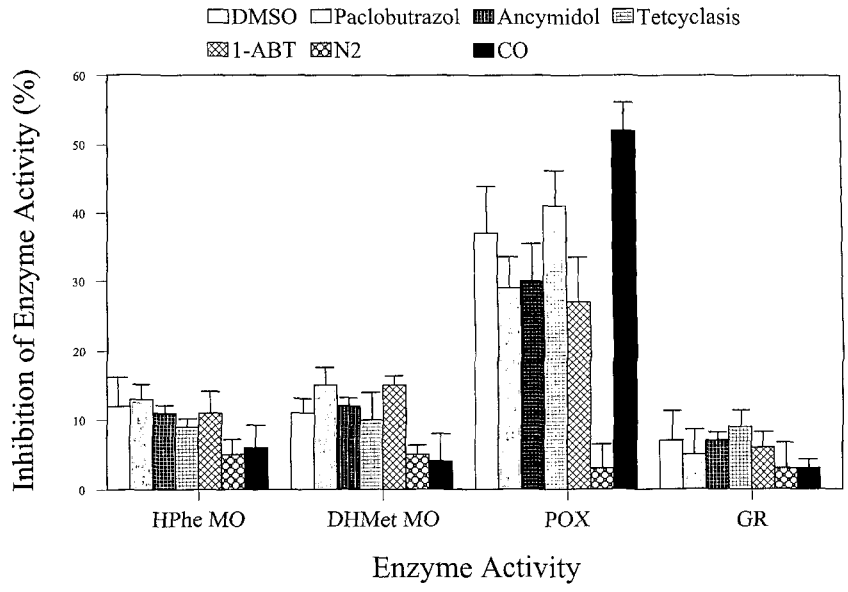

Figure 3. Effects of Cyt P450 inhibitors on enzymes from oilseed rape leaves. See Figure 1 for details. For each enzyme 100% activity was: HPhe MO, 1.055; DHMet MO, 0.93 (all μmol CO_2 mg^{-1} microsomal protein h^{-1}); POX, 67 nmol tetraguaiacol g^{-1} fresh weight h^{-1}; and glutathione reductase (GR), 194 nmol NADPH oxidized g^{-1} fresh weight h^{-1}. 1-ABT, 1-Aminobenzotriazole.

cotyledons after SA treatment (R.N. Bennett, unpublished data).

MO Involved in Glucosinolate Biosynthesis

To identify enzymes involved in glucosinolate biosynthesis it is clearly necessary to look at tissues that manufacture these compounds at a significant rate. Young, expanding true leaves fall into this category (Milford et al., 1989; Clossais-Besnard and Lahrer, 1991; Porter et al., 1991). In young, expanding true leaves of both white mustard and oilseed rape we have detected NADPH-dependent MO activities specific for the precursor amino acids of glucosinolates found in those tissues. Similar MO activities are present in other glucosinolate-containing species but are not detectable in barley, beans, or tobacco, which do not manufacture these secondary metabolites (Bennett et al., 1996). Given the proven involvement of amino acids and aldoximes as intermediates in glucosinolate biosynthesis (Underhill, 1980), it is a reasonable assumption that these aldoxime-forming MOs catalyze the initial step in glucosinolate biosynthesis, and their substrate specificity and developmental regulation support this view (Bennett et al., 1995a, 1995b).

We have used an assay that measures NAD(P)H-dependent $^{14}CO_2$ release from 1-^{14}C-labeled amino acids rather than attempting to identify the presumed aldoxime products, because of the simplicity and sensitivity of our assay and the marked instability of some of the expected aldoximes in the assay mixture (Dawson et al., 1993; R.N. Bennett, G. Kiddle, and R.M. Wallsgrove, unpublished data). The validity of the assay has been demonstrated by the identification of the aldoxime product (Dawson et al., 1993), demonstration of strict substrate specificity (Bennett et al., 1995a), and developmental regulation that mirrors the rate of glucosinolate accumulation (Bennett et al., 1995b). It has also been shown that nonspecific decarboxylases or other amino acid-degrading enzymes are not involved in the reactions we measured (Bennett et al., 1996).

The MO activities detected in *Brassica* sp. are not inhibited by any Cyt P450 inhibitors that we have tested, nor are the HPhe- and DHMet-dependent MOs in white mustard leaves. Our CO inhibition protocol may not saturate the enzymes, because we did not continually gas the reaction vessel, but the same procedure gave good inhibition of the Tyr-dependent MO in white mustard and microsomal peroxidases in extracts from both species (Figs. 2 and 3). A strict O_2 requirement by the oilseed rape MOs has also been demonstrated (Bennett et al., 1995a). These data indicate that earlier criticism of our methods and results (Du et al., 1995) was based on faulty understanding and interpretation. Cyt P450-mediated MO activity in etiolated white mustard cotyledons was reported to be approximately 350 pmol mg^{-1} microsomal protein h^{-1} (Du et al., 1995). In leaf microsome extracts the MO activities with all three substrates are in the range 0.45 to 1.05 μmol mg^{-1} h^{-1} (Figs. 2 and 3). This is consistent with the view that cotyledons do not synthesize alkenylglucosinolates or aromatic glucosinolates (Glen et al., 1990; Bodnaryk, 1991), whereas true leaves manufacture these compounds at high rates during leaf expansion (Porter et al., 1991).

Cyt P450s and Glucosinolate Biosynthesis

Cyt P450s are a varied and heterogeneous family of proteins, with wide variation in characteristics and inhibitor responses (Donaldson and Luster, 1991). Nonetheless, they have some core characteristics that can be used to describe and identify them. First and foremost, they are heme-containing proteins; therefore compounds that interfere with heme groups will inhibit Cyt P450 enzymes. There are a large number of classic Cyt P450 inhibitors known, including CO and tetcyclasis. Biochemically, one diagnostic characteristic of Cyt P450s is the light-reversible

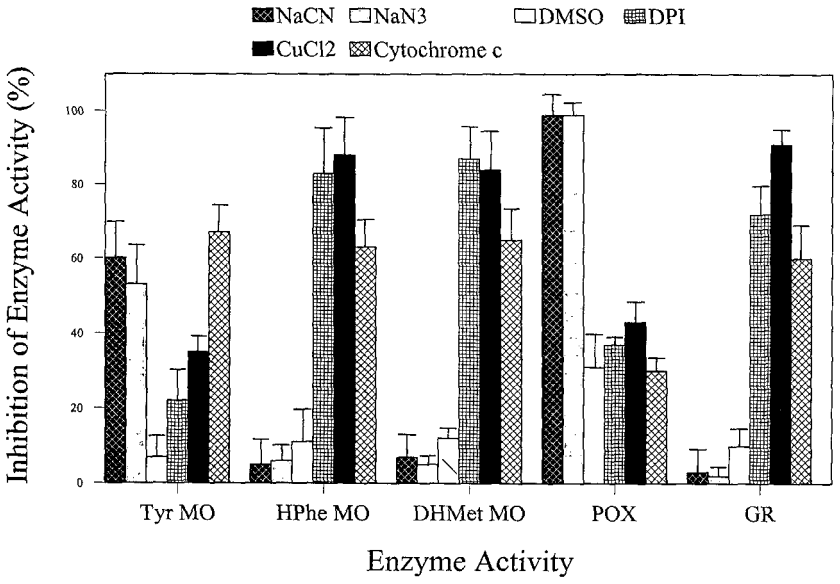

Figure 4. Effects of heme inhibitors (NaCN, NaN_3), a flavoprotein inhibitor (DPI), Cu^{2+}, and Cyt *c* on enzyme activities from white mustard leaves. See Figure 1 for 100% activities. GR, Glutathione reductase.

inhibition by CO, as used to characterize the Tyr-dependent enzyme activity in white mustard (Du et al., 1995). Such light reversibility is almost impossible to demonstrate in green plant extracts because the pigments absorb most of the light, but this test is hardly valid if the enzyme under study is not inhibited by CO in the first place. We have clearly demonstrated in this paper and elsewhere that the MOs active with HPhe and DHMet in oilseed rape and white mustard are not significantly inhibited by CO or any other Cyt P450 inhibitor (Figs. 2–5; Bennett et al., 1993, 1995a, 1996). The same inhibitors used under identical conditions effectively inhibited the Tyr-dependent MO in white mustard, and the Phe-dependent MOs in other (noncruciferous) glucosinolate-containing plants (Bennett et al., 1996, 1997). We can therefore confidently state that the Tyr- and Phe-dependent enzymes in these latter plants are Cyt P450 MOs.

With equal confidence we can state that the other MOs found in crucifers are not Cyt P450s, because apart from their NADPH and O_2 requirement, they share no characteristics with any known Cyt P450 MO. They require different extraction conditions for maximum recovery of activity (Bennett et al., 1996) and in particular are inhibited by DTT (Bennett et al., 1993), which is normally required for the recovery of Cyt P450-catalyzed activities. These *Brassica* sp. MOs are strongly inhibited by DPI and Cu^{2+} ions, as also found for glutathione reductase, a known flavoprotein. These inhibitors do reduce Cyt P450 MO activity in white mustard extracts, presumably by inhibiting the Cyt P450 reductase flavoprotein, but to a much

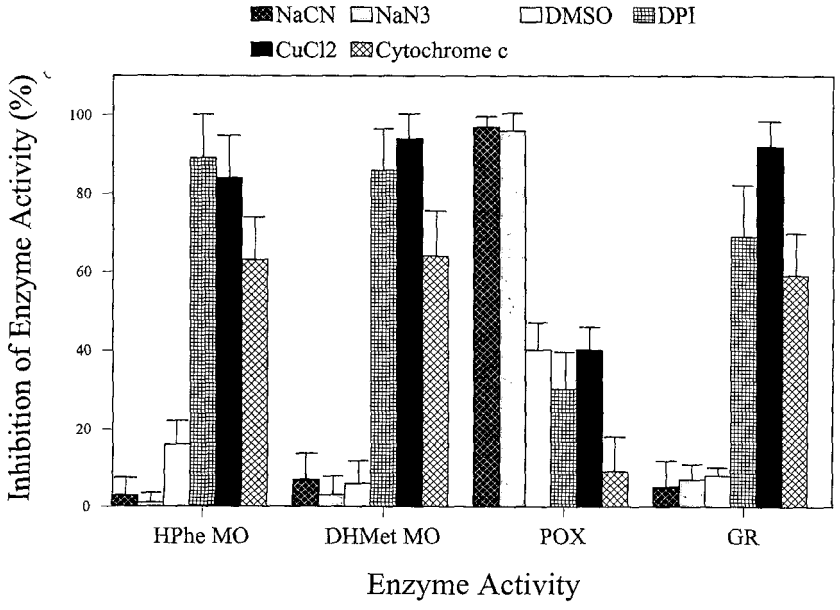

Figure 5. Effects of heme inhibitors (NaCN, NaN_3), a flavoprotein inhibitor (DPI), Cu^{2+}, and Cyt *c* on enzyme activities from oilseed rape leaves. See Figure 2 for 100% activities. GR, Glutathione reductase.

lesser extent. Nonspecific heme inhibitors such as CN and azide have no effect on the *Brassica* sp. MOs. We can thus define these enzymes as having no heme and almost certainly as containing a flavin prosthetic group. We have used appropriate control enzymes (heme-containing peroxidases and flavoprotein glutathione reductase) in the same tissue extracts to ensure that our chosen inhibitors at the concentrations used are both active and specific, and no secondary effects were noted.

Flavoprotein MOs and Glucosinolate Biosynthesis

The crucifer MOs most closely resemble the flavin MOs found in animals and microorganisms, some of which similarly catalyze aldoxime formation (although from amines) (Ziegler, 1988). However, the reaction mechanism may be rather different, because it is highly unlikely that a classic flavin-containing MO could hydroxylate the amino group of an α-amino acid directly. Given that flavoprotein decarboxylases are known (Von Berkel and Müller, 1985) and that amines are prime substrates for flavin MOs (Ziegler, 1988), it is possible to imagine a concerted, two-stage reaction (probably on a single active site) by which a flavoprotein enzyme could convert an amino acid to its aldoxime. An initial decarboxylation (perhaps to an enzyme-bound amine intermediate) would be followed by NAD(P)H- and O_2-dependent N oxidation. We are currently testing this hypothesis.

Glucosinolate Biosynthesis in White Mustard: Biochemical Diversity

The Tyr-dependent Cyt P450 MO reported by Du et al. (1995) has some odd characteristics. It is present in etiolated cotyledons, tissue with little or no active glucosinolate synthesis. The activity was only detectable following treatment of the seedlings with MJ, and yet this elicitor does not stimulate pHBG production in either cotyledons or true leaves of white mustard (Bodnaryk, 1992, 1994; Table I). It is not yet clear whether this enzyme is the same as the very much more active Cyt P450 MO that we have found in the green leaves of white mustard, tissues that actively synthesize pHBG. On the basis of its jasmonate stimulation, it is possible that the cotyledon enzyme is not involved in glucosinolate biosynthesis and represents a different enzyme from that found in green, glucosinolate-accumulating tissues.

The most recent review of the taxonomy of glucosinolate-containing plants (Rodman, 1996) places the Cruciferae in a distinct group, somewhat distant from others. A recent report confirming the presence of an aldoxime-forming Cyt P450 MO in *Tropaeolum majus* (Du and Halkier, 1996) mistakenly assumed that the Tropaeolaceae were within the Capparales order and thus that glucosinolate biosynthesis in *T. majus* was also a model for such synthesis in *Brassica* sp. Neither assumption is correct. Crucifers are particularly characterized by the presence of aliphatic/alkenylglucosinolates derived from Met homologs that are not found in any other families. This distinction is reinforced by the biochemical evidence, which indicates that aldoxime formation for these glucosinolates is catalyzed by flavoprotein MOs, whereas in *Carica*, *Tropaeolum*, and others, aldoxime formation (from Phe or Tyr) is catalyzed by Cyt P450 MOs, as found in cyanogenic plants. Glucosinolate formation in these plants may have evolved from ancestral cyanogenesis, especially in *Carica papaya*, which contains both glucosinolates and cyanogenic glucosides (Bennett et al., 1997), but this would not seem to be true of the Cruciferae. White mustard stands out as an oddity in this analysis because it contains all of the enzymes found in other crucifers and yet has in addition a Tyr-dependent Cyt P450 MO. This is particularly unusual in that other crucifers that contain pHBG do not possess such an enzyme and have instead a flavoprotein MO active with Phe and no Tyr-dependent MO (Bennett et al., 1996).

Evolution of Glucosinolate Biosynthesis

We can thus hypothesize that glucosinolate biosynthesis may have evolved independently at least twice, and one such evolutionary pathway owes little or nothing to ancestral cyanogenesis. White mustard has acquired an additional enzyme to give it the ability to produce sinalbin, possibly by gene duplication. It apparently possesses a minor Tyr-dependent Cyt P450 MO not related to glucosinolate biosynthesis, although other *Brassica* sp. make this same glucosinolate from Phe rather than Tyr. Aldoximes are widely distributed in plants and are involved in many pathways (Mahadevan, 1973). It would not be surprising for aldoxime formation specific for secondary metabolism to be related to other pathways using similar intermediates. This certainly seems to be the case for indole acetaldoxime formation via membrane-bound peroxidases, which are widely distributed in plants and yet are clearly involved in indolylglucosinolate biosynthesis in *Brassica* sp. (Ludwig-Muller and Hilgenberg, 1988; Bennett et al., 1995b). No other enzyme system catalyzing the synthesis of indole acetaldoxime has ever been reported. Although white mustard has very low indolylglucosinolate content, it does contain these compounds and so apparently has three independent and biochemically distinct enzyme systems for aldoxime formation in glucosinolate biosynthesis. Although this makes it very interesting from a biochemical and evolutionary perspective, it means that pHBG formation in this species is an inappropriate model for glucosinolate biosynthesis in other crucifers (at least so far as aldoxime formation is concerned) or indeed for biosynthesis of other glucosinolates in the same tissue.

Progress in isolating key genes for glucosinolate biosynthesis in *Brassica* sp. will unfortunately not be able to make use of the major advances in cloning and molecular characterization of Cyt P450s from plants (Durst and Nelson, 1995). Instead, strategies based on the observed biochemical characteristics of these enzymes need to be used, and we are currently using probes derived from highly conserved regions of NADPH-dependent flavoproteins to search for MO genes in oilseed rape.

ACKNOWLEDGMENT

We gratefully acknowledge the assistance of Alastair Hick in synthesizing substrates and DPI.

Received January 28, 1997; accepted April 30, 1997.
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