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Covalent binding of chloroacetamide herbicides to the active site cysteine of plant type III polyketide synthases

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Abstract

Chloroacetamide herbicides inhibit very-long-chain fatty acid elongase, and it has been suggested that covalent binding to the active site cysteine of the condensing enzyme is responsible [Pest Manage Sci 56 (2000), 497], but direct evidence was not available. The proposal implied that other condensing enzymes might also be targets, and therefore we have investigated four purified recombinant type III plant polyketide synthases. Chalcone synthase (CHS) revealed a high sensitivity to the chloroacetamide metazachlor, with 50% inhibition after a 10 min pre-incubation with 1–2 molecules per enzyme subunit, and the inactivation was irreversible. Stilbene synthase (STS) inactivation required 20-fold higher amounts, and 4-coumaroyltriacetic acid synthase and pyrone synthase revealed no response at the highest metazachlor concentrations tested. A similar spectrum of differential responses was detected with other herbicides that also inhibit fatty acid elongase (metolachlor and cafenstrole). The data indicate that type III polyketide synthases are potential targets of these herbicides, but each combination has to be investigated individually. The interaction of metazachlor with CHS was investigated by mass spectrometric peptide mapping, after incubation of the enzymes with the herbicides followed by tryptic digestion. A characteristic mass shift and MS/MS sequencing of the respective peptide showed that metazachlor was covalently bound to the cysteine of the active site, and the same was found with STS. This is the first direct evidence that the active site cysteine in condensing enzymes is the primary common target of these herbicides.

Keywords: Condensing enzyme; Plant polyketide synthases; Herbicide; Chloroacetamide; Active site; Covalent modification; Chalcone synthase; Stilbene synthase; Pyrone synthase

1. Introduction

Chloroacetamides such as metazachlor and metolachlor (Fig. 1) are used world-wide as pre-emergent herbicides to control annual grasses and many dicotyledonous weeds, predominantly with the crops rice, maize, and rape (reviewed by Fuerst, 1987). Chloroacetamides reduce the amount of very-long-chain fatty acids (VLCFA) in the plasma membrane (Matthes and Böger, 2002) and in epicuticular waxes (Ebert and Ramsteiner, 1984). This causes many secondary effects that finally lead to plant death. The reduction of VLCFAs is the consequence of inhibiting fatty acid elongase (Matthes et al., 1998), the membrane-bound enzyme complex extending long-chain fatty acid CoA-esters by a series of reactions (addition of a C2-unit by condensation with malonyl-CoA, reduction, dehydration, and a second reduction) (see Domergue et al., 1998, for review). In vivo and in vitro studies suggest that the inactivation involves an irreversible reaction with the chloroacetamides, and the proposal was extended to other herbicides that also inhibit fatty acid chain elongation (Böger et al., 2000) (see Fig. 1

Abbreviations: PS, 2-pyrone synthase; CHS, chalcone synthase; CTAL, 4-coumaroyltriacetic acid lactone; CTAS, 4-coumaroyltriacetic acid synthase; PKS, polyketide synthase; STS, stilbene synthase; VLCFA, very-long-chain fatty acids.

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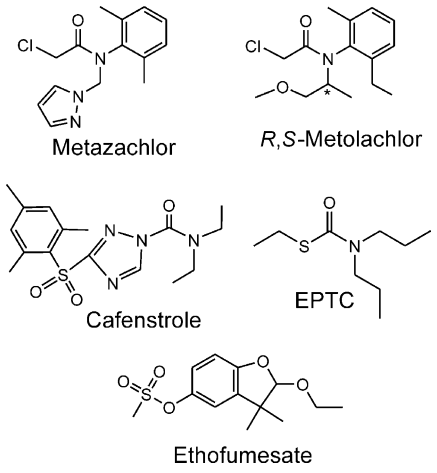

Fig. 1. Structures of the herbicides investigated in this work. Metazachlor, metolachlor (both chloroacetamides), cafenstrole, and ethofumesate act on fatty acid elongation. EPTC (*S*-ethyl dipropylthiocarbamate) is inactive, but becomes an efficient inhibitor after sulfoxidation (reviewed in Böger et al., 2000). The star (*) in metolachlor indicates the chiral carbon atom.

for examples also investigated in our work). Chloroacetamides couple covalently to cysteines in vitro (Levitt and Penner, 1979). All condensing enzymes investigated contain one essential, highly reactive cysteine that covalently binds the starter substrate prior to the condensing reaction, and mutagenesis studies with recombinant proteins show that fatty acid elongases are no exception (Ghanevati and Jaworski, 2001). These considerations led to the proposal that the condensing enzyme in the elongase complex is the primary target of the herbicides, and that the irreversible inactivation reflects the covalent binding of the herbicide to the highly reactive cysteine in the active site (Böger et al., 2000). However, the complexities of working with crude microsomal preparations precluded a rigorous test of the model.

The model implied that other condensing enzymes might also be targets of these herbicides. This is not only of general interest, but might also provide experimental systems that are more suitable to test the validity of the proposal. Excellent candidates are the plant type III polyketide synthases (PKS). They are condensing enzymes with identified active site cysteines (Lanz et al., 1991), and they perform one, two or three sequential condensation reactions leading to a large variety of biologically important secondary products. They constitute a protein family with interesting functional diversities (Schröder, 1999), and the crystal structures of two enzymes (Ferrer et al., 1999; Jez et al., 2000) provided the basis for an initial understanding of the structural reasons for the functional differences (see Austin and Noel, 2003, for a recent discussion).

We have investigated four purified recombinant enzymes from this family (see Fig. 2 for the reactions): (i) the well-known chalcone synthase (CHS) that synthesizes the precursor essential for the biosynthesis of a large number of biologically important compounds (e.g. flavonoids, anthocyanins), (ii) stilbene synthase (STS), an enzyme present in relatively few plants, (iii) 4-coumaroyltriacetic acid synthase (CTAS), a protein from *Hydrangea macrophylla* using the same substrates as CHS and STS, but synthesizing other products (Eckermann et al., 2003), and (iv) a pyrone synthase (2PS)

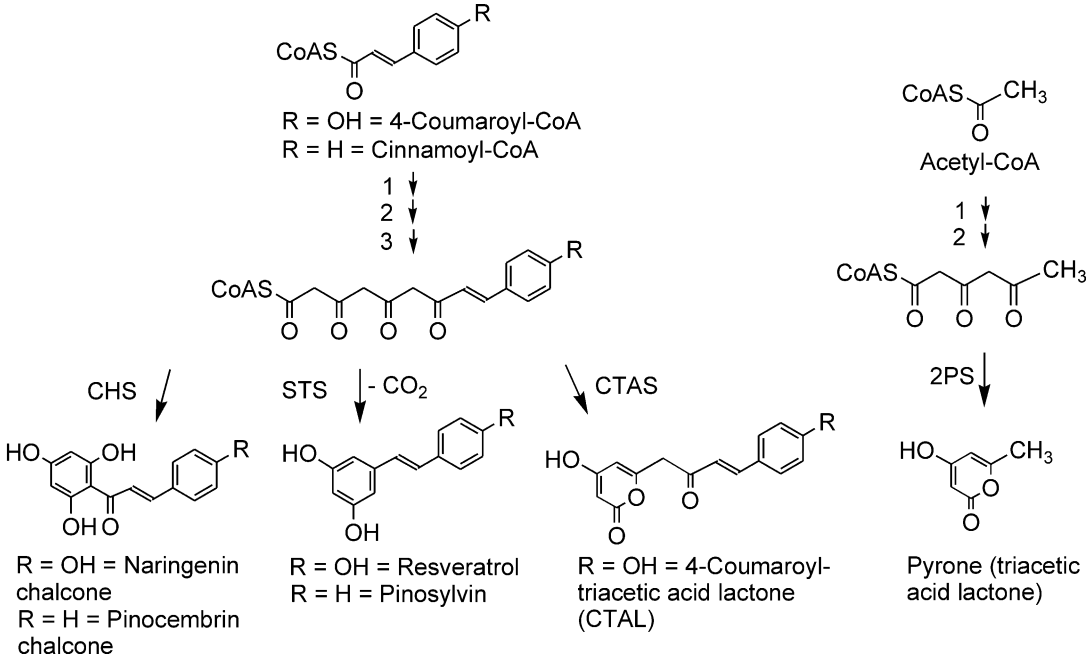

Fig. 2. Substrates, key intermediates, and products of four type III plant polyketide synthases. CHS, chalcone synthase; STS, stilbene synthase; CTAS, 4-coumaroyltriacetic acid synthase; 2PS, pyrone synthase. 1, 2, and 3 indicate the number of condensation reactions.

from *Gerbera hybrida* that produces the backbone for an unusual phytoalexin (Eckermann et al., 1998). The in vivo products indicate that CHS, STS, and CTAS use phenylpropanoid-CoA esters as substrates and perform three condensation reactions, while the 2PS uses acetyl-CoA and performs only two condensations. However, all are known to accept a broad range of substrates in vitro, indicating a considerable flexibility in the active site.

The results with several herbicides reveal a complex spectrum of effects, ranging from very high sensitivity to no response. With two of the enzymes it could be unambiguously demonstrated that the chloroacetamide herbicide metazachlor couples covalently and specifically to the active site cysteine of the proteins.

2. Results and discussion

2.1. The chloroacetamide herbicide metazachlor inactivates CHS irreversibly

Metazachlor added directly to CHS incubations is not an effective inhibitor as 0.3 mM are necessary to achieve a 50% reduction of the enzyme activity. However, the sensitivity increases dramatically if the enzyme is pre-incubated for 10 min with the herbicide, and under these conditions 0.3 μM are sufficient to reduce the activity by half. Including the substrate 4-coumaroyl-CoA in the pre-incubation protects against inactivation, suggesting that it hinders the access or/and a reaction of the herbicide with the protein. Addition of the substrate after pre-incubation does not reverse the inactivation, as shown by the following experiment. The enzyme is pre-incubated for 10 min with increasing concentrations of metazachlor (0.0, 0.1, 0.3, and 0.5 μM), and the subsequent activity measurements are performed with increasing concentrations of 4-coumaroyl-CoA (8, 10, 12, 14, or 16 μM) and 30 μM ^{14}C-malonyl-CoA (chain extender). The results confirm that the inactivation is dependent on the metazachlor concentration, and more importantly, they show that it is irreversible because a re-activation is not possible even with a 160-fold excess of 4-coumaroyl-CoA to the herbicide (16 μM 4-coumaroyl-CoA against 0.1 μM metazachlor).

The time dependence of the inactivation was determined in the next series of experiments. The CHS was pre-incubated with increasing metazachlor concentrations (molar ratio of enzyme to herbicide = 0 to 2.0) for increasing times prior to the start of the enzyme reaction by addition of the substrates. The analysis (Kitz and Wilson, 1962) shows that the inactivation is complete within 8 – 10 min (Fig. 3). The experimental data indicated a slow, time-dependent, and irreversible binding mechanism because the velocity constant for the back reaction can be neglected. The *x*-intercept in the lower graph of Fig. 3 represents the I_{50}-value. It was calculated to 0.14 μM which is in good agreement with the 0.3 μM determined in the other experiments.

2.2. The inactivation is compound-specific and not all type III plant PKSs are sensitive

These experiments investigate whether the results obtained with metazachlor and CHS can be generalized with respect to the herbicide type and with respect to other PKS type III enzymes. If the herbicides indeed

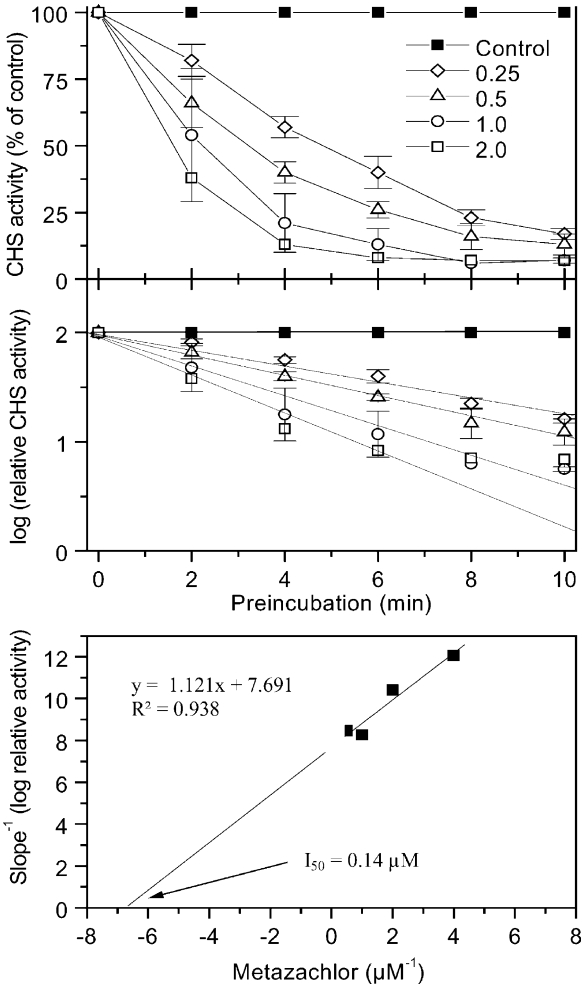

Fig. 3. Time dependence of CHS inactivation in the pre-incubation, and dependence on varying metazachlor/CHS ratios. The pre-incubations were carried out with 18 μM CHS (calculated as monomers) and metazachlor concentrations of 4.5, 9, 18, and 36 μM (resulting in the ratios of enzyme to herbicide shown in the inset of the top graph). After the pre-incubation times indicated, the enzyme was diluted 40-fold into activity assays containing 20 μM 4-coumaroyl-CoA and 30 μM ^{14}C-malonyl-CoA, and the CHS activity was determined. The dilution and the presence of the high 4-coumaroyl-CoA concentration (see Results) protected the enzyme against further inactivation after the pre-incubation. Top: CHS inactivation dependence on the length of pre-incubation and on the ratio herbicide to enzyme. Middle: data on the logarithmic scale, and bottom: analysis according to Kitz and Wilson (1962).

Table 1
Inhibition of four enzymes from the family of CHS related proteins (type III PKSs) by herbicides (see Fig. 1 for the structures). The purified proteins were pre-incubated for 10 min at 30 °C prior to the start of the enzyme reaction by addition of the substrates (10 μM starter CoA-ester, 15 μM ^{14}C-malonyl-CoA)

Compound	I_{50} (μM)[a]			
	CHS[b] (0.25 μM)	STS[c] (0.125 μM)	CTAS[d] (0.75 μM)	2PS[e] (0.5 μM)
Metazachlor	0.3	7–9	>100	>100
R-Metolachlor	5	10	>100	>100
S-Metolachlor	5	10	>100	>100
Cafenstrole	5	0.5	>100	>100
Ethofumesate	>100	>100	>100	>100
EPTC	>100	>100	>100	>100

[a] Calculated from duplicate incubations with 0.1, 1, 10, and 100 μM herbicide. >100 = no significant inhibition at the highest concentration tested.
[b] Substrate 4-coumaroyl-CoA, product naringenin.
[c] Substrate cinnamoyl-CoA, product pinosylvin.
[d] Substrate 4-coumaroyl-CoA, product 4-coumaroyltriacetic acid lactone.
[e] Substrate benzoyl-CoA, products phenylpyrone (from benzoyl-CoA) and triacetic acid lactone (from acetyl-CoA produced by decarboxylation of malonyl-CoA) (Eckermann et al., 1998).

bind to the active site cysteine, they must have access to it. It is at the bottom of a deep cleft in these PKSs, and the size of the active site pocket is determined by the size of the substrate and the number of condensation reactions (Ferrer et al., 1999; Jez et al., 2000). CHS, STS, and CTAS have the same substrate range in vitro, and all perform three condensations. It was therefore expected that STS and CTAS reveal similar sensitivities as CHS. The results, however, reveal an unexpected broad range of responses (Table 1).

Metazachlor is confirmed as a very potent inactivator of CHS, with little more than one molecule sufficient to inactivate one enzyme molecule. The concentrations are comparable to those observed with fatty acid chain elongase assays in vitro (Schmalfuß et al., 2000; Böger et al., 2000), but the use of crude microsomes precludes a precise determination of the target protein number in such cases. The high sensitivity qualifies the CHS as a potential primary target (see definition in Böger et al., 2000). However, this is not true for the other PKSs as they are much less sensitive (stilbene synthase, STS) or reveal no significant reaction at the highest concentrations tested (CTAS and 2PS). The STS requires about 20-fold higher concentrations than CHS for 50% inactivation. Fig. 4 compares the data for CHS and STS and demonstrates that the inactivation of PKS is accompanied by a large increase in the activity of decarboxylating the chain extender malonyl-CoA to acetyl-CoA. The same finding has been reported for iodoacetamide, an efficient inhibitor of both CHS and STS (I_{50} = 0.3–0.5 μM) (Eckermann et al., 2003). The iodoacetamide-induced conversion into malonyl-CoA decarboxylases is typical for condensing enzymes; it is a consequence of the covalent carboxamidomethylation of the HS-group in the cysteine binding the starter substrate (see for example Dimroth et al., 1976; Kresze et al., 1977; Shoolingin-Jordan and Campuzano, 1999; Austin and Noel, 2003). These similarities between metazachlor and iodoacetamide suggest that both have the same mechanism.

The large difference in sensitivity to metazachlor observed for CHS and STS remains puzzling. Both accept in vitro the same range of substrates (Fliegmann et al., 1992; Schröder, 1999, 2000; Eckermann et al., 2003), and the different substrates chosen here for CHS

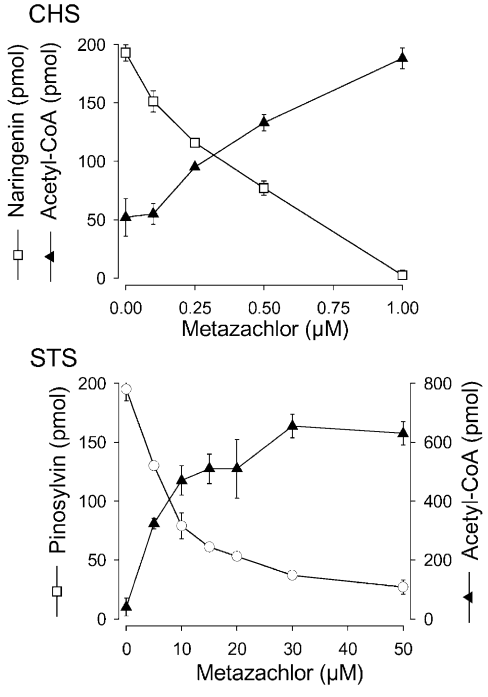

Fig. 4. Differential inhibition of CHS and STS by metazachlor, and conversion of the enzymes into malonyl-CoA decarboxylases by the herbicide (stimulation of acetyl-CoA formation). The activities were determined under the standard assay conditions (see Experimental), with 4-coumaroyl-CoA and cinnamoyl-CoA as starter CoA-esters for CHS and STS, respectively.

and STS reflect the use of the physiological substrates, but there is not a real difference in substrate preference. The important difference between CHS and STS is the type of ring-folding to the final product (chalcone versus stilbene, Fig. 2), and it is tempting to speculate that the different sensitivities are a consequence of this functional difference.

It is remarkable that metazachlor has no significant effect on CTAS. This is not easily understood if only the substrate or number of condensations are considered as CTAS, CHS, and STS have very similar substrate preferences (Eckermann et al., 2003), and all three carry out three condensations. However, CTAS is functionally clearly different as it synthesizes a third product type (CTAL, Fig. 2). Recent results revealed moreover that CTAS synthesizes a fourth type of product from dihydro-4-coumaroyl-CoA (a stilbenecarboxylate, not shown), and other properties also distinguish CTAS from CHS and STS (Eckermann et al., 2003). Again, these data suggest that functional aspects influence the sensitivity, but the mechanisms remain to be explored. The lack of response with 2PS (Table 1) is more easily rationalized because large CoA-esters (e.g. 4-coumaroyl-CoA or cinnamoyl-CoA) are not accepted as substrates (Eckermann et al., 1998), and the crystal structure of 2PS and mutagenesis studies showed that benzoyl-CoA, the substrate used in our experiments, is probably of the maximum size that can be accommodated in the active site (Jez et al., 2000). It seems likely that metazachlor is too bulky to enter the active site pocket of 2PS, in contrast to the small iodoacetamide that is an effective inhibitor of 2PS (Eckermann et al., 2003). The same reasoning most likely applies to all other herbicides investigated in this study with 2PS (Table 1).

Metolachlor, a chloroacetamide with some similarity to metazachlor (Fig. 1), also inactivates CHS and STS. CHS does not react with the high sensitivity observed with metazachlor, but the inactivation of the four PKSs follows the same tendency as observed with metazachlor (Table 1), e.g. with CTAS and 2PS very unresponsive to the herbicide. Metolachlor occurs in two enantiomers, and only the *S*-enantiomer is phytotoxic and herbicidal (Moser et al., 1982). The *S*-, but not the *R*-enantiomer, inhibits the chain elongation step in the biosynthesis of VLCFAs, and that is a major point in the identification of this reaction as a physiological target of the herbicide (Schmalfuß et al., 1998, 2000). It was therefore of interest to determine whether the CHS possesses the same enantiomer specificity. Table 1 and Fig. 5 show that CHS reveals no such clear discrimination, indicating that it is probably not a primary target of this herbicide.

Cafenstrole, a carbamoylsulfonyltriazole herbicide and potent inhibitor of fatty acid elongase (Takahashi et al., 2001) is also thought to form a covalent bond with active site cysteines (Böger et al., 2000). The effects with the four PKSs clearly follow the same trend as with metazachlor and metolachlor (Table 1) because CHS and STS are sensitive, while CTAS and 2PS reveal no significant effects at the highest concentrations tested. It is remarkable that in this case STS is more sensitive than CHS, raising again the question whether this might reflect functional differences between the two enzymes. The sum of the results, however, supports the proposal that this herbicide acts on condensing enzymes.

Two other herbicides investigated in our work, EPTC and ethofumesate (Fig. 1), reveal no significant effects with any of the four PKSs (Table 1). Previous experiments with cafenstrole suggest an explanation for the result with EPTC: Takahashi et al. (2001) showed that complete oxidation of the sulfur in cafenstrole (Fig. 1) is prerequisite for inhibition of VLCFA elongation. The sulfur is not oxidized in EPTC, and, as the in vitro assay conditions (see Böger et al., 2000) do not lead to oxidization of the sulfur, this could explain the lack of effect with EPTC. The result with ethofumesate, however, is unexpected because the compound is an inhibitor of fatty acid elongases (Abulnaja et al., 1992). It is possible that this molecule simply does not fit into the active site pockets of the PKSs, but that needs to be investigated further.

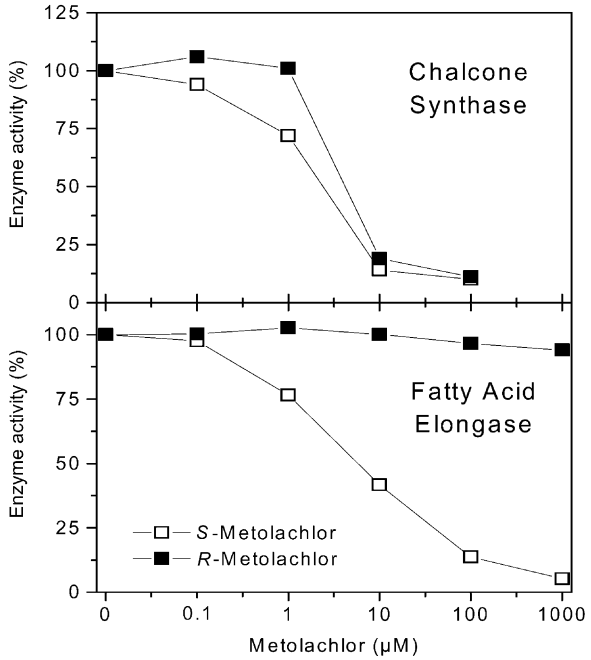

Fig. 5. CHS and fatty acid elongase react differently to different isomers of metolachlor. The enzyme preparations (purified recombinant CHS; fatty acid elongase in a microsomal preparation from *Allium porrum*) were incubated for 10 min with either *R*- or *S*-metolachlor, and the enzyme activities were then started by addition of the substrates. The incubations were performed for 20 min at 30 °C using [^{14}C]malonyl-CoA and stearoyl-CoA (fatty acid elongase), or 4-coumaroyl-CoA (CHS). In absence of the herbicide, the CHS activity was 1.1 ± 0.1 μmol mg^{-1} protein h^{-1} and that of the fatty acid elongase was 10.5 ± 1.5 nmol mg^{-1} protein h^{-1}.

2.3. Metazachlor binds covalently to the active site cysteine of chalcone and stilbene synthase

The high sensitivity of CHS to metazachlor, the kinetics, and the irreversibility of the inactivation support the proposal of covalent binding. This was directly tested by incubations of the enzyme with radioactive metazachlor, followed by various treatments to remove non-covalently bound herbicide (e.g. treatments with urea, guanidinium hydrochloride, 2 M NH_4OH, or standard SDS-PAGE sample buffer, see Experimental for details). The autoradiography after SDS-PAGE of the treated samples showed that the protein band contains radioactivity not removed by any of these procedures, and treatment with 3 M HCl is necessary to release the radioactivity. The same binding conditions fail to show any binding of radioactivity to protoporphyrinogen oxidase from chicory (Adomat and Böger, 2000), bovine serum albumin, or phenylalanine ammonium-lyase (Sigma, Deisenhofen, Germany). The data strongly support that the herbicide binds specifically and covalently to the PKS. Fig. 6a shows as one example the time dependence of metazachlor-binding to CHS. It is complete within 5 to 10 min, and this agrees well with the time dependence of the inactivation.

The binding site was investigated by experiments in which the CHS, after incubation with metazachlor, is digested with trypsin, and the peptides are subjected to MS and MS/MS analysis. Fig. 6b shows the protein sequence of the CHS aligned with the STS used in our work. The CHS sequence predicts eight cysteine-containing tryptic peptides, with the peptide containing the active site cysteine (underlined in Fig. 6b) clearly different from all others. Fig. 6c shows the structure predicted for the cysteine derivative with metazachlor.

Based on the inactivation studies, the CHS incubations with metazachlor were carried out with approximately equimolar concentrations of protein and herbicide (about 25 μM). We then added a 20-fold excess (0.5 mM) of another compound known to react covalently with free sulfhydryl groups. Iodoacetamide was chosen because recent experiments (Eckermann et al., 2003) show that one or two molecules are sufficient to inactivate all enzymes used in our investigation, and thus it was expected that the excess iodoacetamide would efficiently inhibit further reactions with metazachlor. The masses of the unmodified and those expected for the modified peptides are listed in Table 2.

The ESI-MS analysis of the tryptic digest after incubation with the herbicide described above reveals a doubly charged peptide with the mass expected from the covalent modification of the active site cysteine by metazachlor at m/z 1001.5 $[M+2H]^{2+}$ (data not shown), and the MS/MS analysis of this parent ion shows a fragmentation pattern clearly demonstrating that the only cysteine in this peptide is the modified residue (Fig. 7a, see legend for explanation). The same preparation also contains the doubly charged peptide $[M+2H]^{2+}$ covalently modified by iodoacetamide at m/z 909.5, as shown by MS/MS analysis (Fig. 7b). This is expected because the 1:1 ratio of CHS and metazachlor (about 50% inhibition, Table 1) was chosen for the identification of selective preferential binding, not for saturation of the protein. A complete and quantitative coverage of all peptides is very difficult in this type of analysis, and therefore it is not possible to establish a clear time course for the metazachlor binding. We also analyzed an experiment carried out with a 5-fold excess of metazachlor and without quenching by iodoacetamide and without removal of the herbicide prior to the tryptic digestion. The MS analysis reveals that all cysteine containing peptides can be modified with metazachlor under these conditions. Given the specificity observed in the other experiment, it seems likely that the majority of these reactions occurred during or

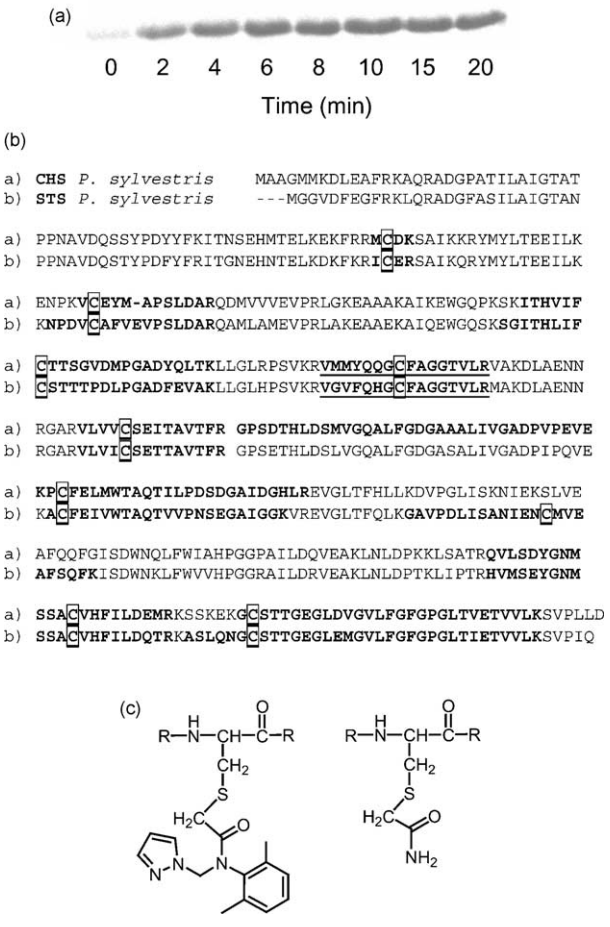

Fig. 6. Covalent binding of metazachlor to CHS. (a) Time course of CHS labelling with radioactive ^{14}C-metazachlor. The figure shows the autoradiography obtained after SDS gel electrophoresis. (b) Sequences of the CHS and the STS from *P. sylvestris*. Tryptic peptides containing cysteines are bold, and the cysteines are boxed. Underlined, tryptic peptides containing the active site cysteine. (c) Predicted structure of cysteine adducts with metazachlor (left) and iodoacetamide (right).

Table 2
Tryptic peptides of CHS and STS containing the active site cysteine (boxed), and expected *m*/*z* values of the unmodified and modified peptides

Enzyme	Tryptic peptide containing the active site cysteine	Expected *m*/*z* values		
		Unmodified	+ Metazachlor	+ Iodoacetamide
CHS	VMMYQQG[C]FAGGTVLR	1760.8	2001.9	1817.9
STS	VGVFQHG[C]FAGGTVLR	1647.8	1888.8	1704.8

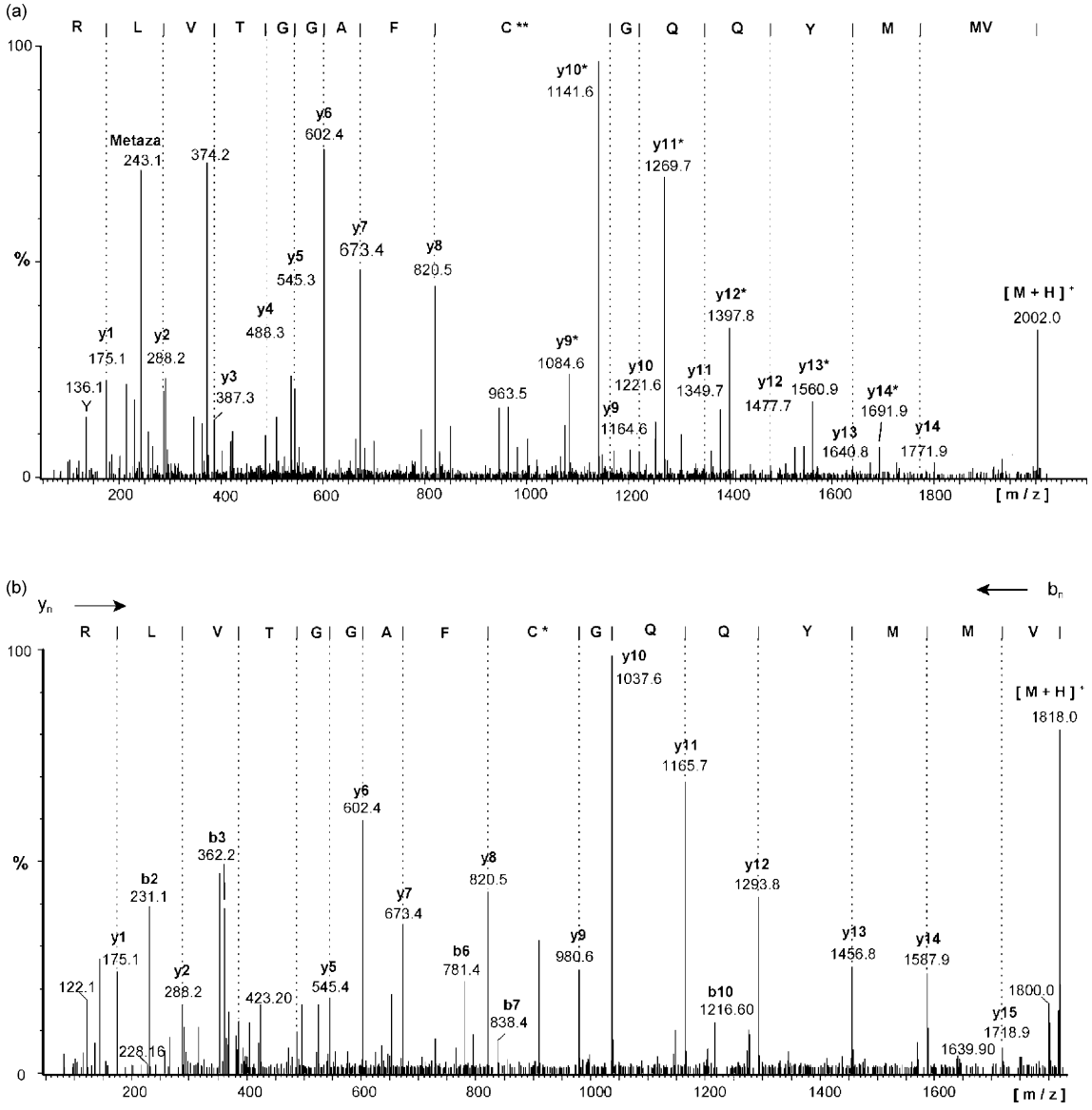

Fig. 7. MS/MS analysis of the tryptic peptides from CHS containing the active site cysteine covalently modified by metazachlor (a) or iodoacetamide (b). Computer enhanced (Max. Ent. 3, Micromass, Manchester, Great Britain) daughter ion spectrum of the doubly charged molecular ion of the tryptic peptides. The sequence of the carboxamidomethylated peptide could be easily deduced from the carboxyterminal fragment ion series of the y-type showing the expected mass increments compared to the unmodified peptide after position 9. In the case of the metazachlor-derivatized peptide the fragment ions y1–8 were identical indicating the same unmodified carboxyterminal peptide sequence. The primary fragments of the y-series incorporating the herbizide derivatized C residue (y9–14) were rather weak, but clearly detectable. Additionally, a much more intense secondary sequence specific fragment ion series probably generated by the elimination of the pyrazol-side chain of the herbizide (−80 Da corresponding to the loss of $C_4H_4N_2$) was observed (marked by asterisks). C**: cysteine modified by metazachlor; C*: cysteine modified by iodoacetamide.

after the tryptic digestion, but nevertheless the result suggests that the selectivity observed in the other experiment does not simply reflect a general inaccessibility or non-reactivity of the other cysteines.

The same experiments were performed with the STS. In these experiments a ratio of 8:1 between herbicide and protein was used, based on the result that much higher concentrations of metazachlor are necessary for STS than for CHS inactivation (Table 1), and the reaction was terminated by iodoacetamide addition. The position of the active site cysteine in STS corresponds to that in CHS (Fig. 6B), but the sequence differences lead to different masses (Table 2). The analysis by MS and MS/MS identify the active site cysteine as the main target of covalent modification by metazachlor and iodoacetamide (not shown). The results also reveal at least one other cysteine containing peptide modified by the herbicide. It seems unlikely, however, that such additional modification contributes significantly to the enzyme inactivation because STSs, like CHSs, contain only one cysteine essential for enzyme activity (Lanz et al., 1991).

2.4. Concluding remarks

This is the first direct evidence that chloroacetamide herbicides inactivate condensing enzymes by covalent modification of the active site cysteines. It is therefore an interesting question whether fatty acid elongases are the only major targets in vivo. According to proposed criteria (Böger et al., 2000) the high sensitivity of CHS to metazachlor and of STS to cafenstrole in vitro (Table 1) qualify these enzymes as potential primary targets. It would be interesting to see whether this is not only an in vitro effect, i.e. whether the herbicides indeed reduce the formation of the natural products synthesized via these enzymes. The few available data on the effects of the chloroacetamide alachlor on anthocyanin formation appear to be conflicting and not conclusive (Molin et al., 1986, 1990; Alla and Younis, 1995). All other herbicide/enzyme combinations tested (Table 1) fail to reveal the required high sensitivity, and with metolachlor the lack of differential response to the two enantiomers (Fig. 5) confirms that the herbicide-specific effects are probably not caused by inactivation of CHS.

The complexity of the interactions between the different PKSs and the various herbicides is unexpected. It clearly suggests that a simple model expecting similar sensitivities for enzymes with the same substrate range and three condensation reactions (CHS, STS, CTAS) is too simplistic. Understanding these differences on a protein structural level might provide interesting information on the fine-tuning of the enzymes for the synthesis of the different products, and experiments with related compounds may have the potential for very sensitive recognition of structural and/or functional differences in this family of proteins.

3. Experimental

3.1. Recombinant proteins and enzyme activity determinations

The expression cloning and purification of the recombinant proteins have been reported: CHS from *Pinus sylvestris* (Schröder et al., 1998), STS from *Pinus sylvestris* (Eckermann et al., 2003), 2PS from *Gerbera hybrida* (Eckermann et al., 1998; Jez et al., 2000), and CTAS from *Hydrangea macrophylla* (Eckermann et al., 2003). Standard assays (0.1 ml) contained 50 mM HEPES-KOH (pH 7), 10 μM starter CoA-ester, 15 μM [2-^{14}C]malonyl-CoA (70,000 dpm), and 0.1–0.75 μM enzyme (according to activity, calculated as dimers). The incubations were for 15 min at 30 °C and terminated by acidification (5 μl 10% acetic acid). The quantitative determination of the polyketide products (TLC analysis of ethyl acetate extracts) and of the acetyl-CoA formed in the incubation (TLC analysis of the aqueous phase) have been described (Eckermann et al., 2003). In the experiments with herbicides, the enzymes were incubated for 10 min at 30 °C before the start of the reaction by addition of the substrates. Additional details or modifications are given in the text or in the figure legends.

3.2. Binding of radioactive metazachlor to CHS

The incubations (10 μl) contained 50 mM HEPES-KOH (pH 7.5), 10 μM CHS, and 10 μM [^{14}C]metazachlor (200 kBq, 2.3×10–12 Bq/mol, a gift from BASF AG, Limburgerhof). Standard incubations were for 30 min at 30 °C, and they were terminated by adding SDS-PAGE buffer and heating for 10 min to 95 °C. The samples were subjected to SDS-PAGE, and the protein-bound radioactivity was estimated by densitometric analysis of the autoradiography films. Other studies of the binding involved after the incubation a treatment with 4 M guanidinium hydrochloride, 4 M urea, 2 M NH_4OH, or 3 N HCl for 30 min at 60 °C. In these cases the reagents were removed with published techniques prior to the SDS-PAGE (Wessel and Flügge, 1984).

3.3. Fatty acid elongase

The activity was determined in a microsomal fraction of *Allium porrum*, measuring the formation of arachidic acid (C20:0) from stearoyl-CoA (C18:0-CoA) and [^{14}C]malonyl-CoA (Schmalfuß et al., 2000).

3.4. MS analysis of tryptic peptides

The binding assays for CHS contained 27 μM CHS and 25 μM metazachlor in a final volume of 25 μl. They were terminated after 10, 20, 30, or 60 min by adding

iodoacetamide to a final concentration of 0.5 mM; this derivatized the remaining free sulfhydryl groups. The assays with STS were identical except for higher metazachlor concentrations (200 μM), based on the low sensitivity of the enzyme.

The electrospray ionization tandem mass spectrometry (ESI-MS/MS) was carried out as follows. A portion (3 μl) of the peptide solution generated by the trytic digestion of the proteins were filled into a gold-coated nanospray glass capillary (Protana, Odense, Denmark). The tip of the capillary was placed orthogonally in front of the entrance hole of a quadrupole time-of-flight (QTOF 2) mass spectrometer (Micromass, Manchester, Great Britain) equipped with a nanospray ion source, and a voltage of approximately 1000 V was applied. For collision-induced dissociation experiments, parent ions were selectively transmitted from the quadrupole mass analyser into the collision cell. Argon was used as the collision gas and the kinetic energy was set at around −35 eV. The resulting daughter ions were then separated by an orthogonal time-of-flight mass analyser.

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