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Mechanism-Based Inactivation of Human Cytochrome P450 2B6 by Chlorpyrifos

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Abstract

Chlorpyrifos (CPS) is a commonly used pesticide which is metabolized by P450s into the toxic metabolite chlorpyrifos-oxon (CPO). Metabolism also results in the release of sulfur, which has been suggested to be involved in mechanism-based inactivation (MBI) of P450s. CYP2B6 was previously determined to have the greatest catalytic efficiency for CPO formation *in vitro*. Therefore, we characterized the MBI of CYP2B6 by CPS. CPS inactivated CYP2B6 in a time- and concentration-dependent manner with a k_{inact} of 1.97 min^{-1} , a K_I of $0.47 \mu\text{M}$, and a partition ratio of 17.7. We further evaluated the ability of other organo-phosphate pesticides including chlorpyrifos-methyl, diazinon, parathion-methyl, and azinophos-methyl to inactivate CYP2B6. These organophosphate pesticides were also potent MBIs of CYP2B6 characterized by similar k_{inact} and K_I values. The inactivation of CYP2B6 by CPS was accompanied by the loss of P450 detectable in the CO reduced spectrum and loss of detectable heme. High molecular weight aggregates were observed when inactivated CYP2B6 was run on SDS-PAGE gels indicating protein aggregation. Interestingly, we found that the rat homologue of CYP2B6, CYP2B1, was not inactivated by CPS despite forming CPO to a similar extent. On the basis of the locations of the Cys residues in the two proteins which could react with released sulfur during the metabolism of CPS, we investigated whether the C475 in CYP2B6, which is not conserved in CYP2B1, was the critical residue for inactivation by mutating it to a Ser. CYP2B6 C475S was inactivated to a similar extent as wild type CYP2B6 indicating that C475 is not likely the key difference between CYP2B1 and CYP2B6 with respect to inactivation. These results indicate that CPS and other organophosphate pesticides are potent MBIs of CYP2B6 which may have implications for the toxicity of these pesticides as well as the potential for pesticide–drug interactions.

Graphical Abstract

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INTRODUCTION

Chlorpyrifos (*O,O*-diethyl-*O*-[3,5,6-trichloro-2-pyridyl] phosphorothionate; CPS), an organothiophosphate (OP) insecticide, is commonly used for agricultural and commercial purposes. It was also approved for residential use until 2002 when such uses were banned by the United States Environmental Protection Agency (USEPA).¹ CPS acts as a neurotoxicant by inhibiting acetylcholinesterase activity. In addition, CPS has also been shown to inhibit neuronal development at levels lower than those associated with its inhibition of acetylcholinesterase^{2,3} leading to developmental delays,^{4–6} and exposure may be related to Gulf war syndrome.⁷ CPS is currently under registration review by the USEPA. As part of that process, the USEPA has identified that certain populations, such as occupational workers and bystanders near treated crops, are exposed to CPS at levels that could cause adverse effects.^{8,9}

Like other OP pesticides, CPS itself is a relatively weak inhibitor of acetylcholinesterase and needs to undergo oxidative desulfurization by cytochrome P450s (P450) to the more potent oxon metabolite chlorpyrifos-oxon (CPO).¹⁰ P450s also perform dearylation of CPS forming the detoxification product 3,5,6-trichloro-2-pyridinol (TCP).¹⁰ Both desulfurization and dearylation by P450s are proposed to occur following the formation of an unstable phosphooxythiran intermediate (Scheme 1). Multiple human P450s can metabolize CPS; however, they differ considerably in their catalytic efficiency and the relative amounts of each metabolite that is formed. CYP2B6, CYP2C19, and CYP3A4 are the major human isoforms that metabolize CPS.^{11–14} CYP2B6 forms predominantly the toxic metabolite CPO and has the highest efficiency for desulfuration of CPS with a k_{cat}/K_m of $15.6 \mu\text{M}^{-1} \text{min}^{-1}$.^{11,15} In contrast, CYP3A4 produces equal amounts of CPO and TCP, while CYP2C19 produces primarily TCP.¹¹ On the basis of a study using human liver microsomes, it is likely that CYP2B6 is the human P450 that will play the major role in CPO formation in humans, especially at toxicologically relevant concentrations.¹⁴

Previous studies have indicated that OP insecticides are capable of mechanism-based inactivation of P450s following oxidative desulfuration.^{16–20} Inactivation is believed to occur following the release of a sulfur molecule during desulfuration which then binds to the P450 primarily at Cys residues.¹⁶ Inactivation is accompanied by loss of detectable heme, loss of a detectable P450-CO complex, and protein aggregation.¹⁶ Despite the knowledge that animal and human P450s are inactivated by OP pesticides, little work has been done to assess the human relevance of inactivation despite the fact that OPs are widely

used resulting in significant human exposures. A critical step in predicting if inactivation would occur in humans at environmentally relevant concentrations and what the potential consequences would be for the metabolism of other xenobiotics is the determination of the kinetic parameters for the inactivation. Inactivation of the human P450s that primarily metabolize CPS could have potential implications for drug metabolism as these enzymes are responsible for the metabolism of a large number of clinically used drugs. Inactivation could also alter the metabolism of other xenobiotics including the OP pesticides during subsequent exposures. This could have significant consequences as CPS is believed to be bioactivated locally in the brain^{6,21} and reduced first-pass metabolism could result in higher levels reaching the brain.

In this study, we investigated the inactivation of CYP2B6 by CPS for three major reasons: (1) CPS is one of the most widely used pesticides in the world, (2) CYP2B6 has the highest catalytic efficiency among human P450s tested for the desulfuration of CPS *in vitro*, and (3) studies suggest that CYP2B6 is likely to play a major role in the oxidative metabolism of CPS in humans. We have determined the kinetics for the inactivation of CYP2B6 by CPS, and we have performed a detailed investigation of the inactivation in order to confirm whether previous mechanistic findings determined with animal P450s and OPs were similar for the inactivation of human CYP2B6 by CPS and provide further elucidation of the mechanism of inactivation. The key new findings of this study include determination of k_{inact} and K_I values for the inactivation of CYP2B6 by multiple OPs, which indicate that OPs are very potent inactivators of CYP2B6; the demonstration that the homologous rat CYP2B1 is not inactivated by CPS; and that the difference in the ability of these two enzymes to be inactivated does not appear to be due to the additional Cys residue present in CYP2B6 (C475). Furthermore, our mechanistic studies demonstrate that heme loss is the major mechanism leading to the inactivation of CYP2B6 by CPO.

MATERIALS AND METHODS

Caution:

OP pesticides and their oxon metabolites are hazardous and should be handled with care. CPS, CPS-methyl, diazinon, azinophosmethyl, parathion-methyl, CPO, and CPS-methyl oxon were handled using the proper personal protective equipment, and all solutions of these pesticides were prepared in an appropriately ventilated chemical fume hood.

Chemicals.

CPS, CPS-methyl, diazinon, azinophos-methyl, parathion-methyl, CPO, CPS-methyl oxon, and TCP were purchased from Chem Service Inc. (West Chester, PA). β -Mercaptoethanol, NADPH, DTT, GSH, DLPC, and catalase were purchased from Sigma-Aldrich Inc. (St. Louis, MO). 7-Ethoxy-4-trifluoromethylcoumarin (7-EFC) was purchased from Invitrogen (Carlsbad, CA). Carbon monoxide with purity >99.5% was purchased from Cryogenic Gas (Detroit, MI). All other chemicals were reagent grade purchased from commercial sources.

Mutagenesis, Overexpression, and Purification of CYP2B6.

Site-directed mutagenesis was performed to prepare the C152S and C475S mutants using the QuikChange method according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). A pair of mutagenic primers, 5'-ATTCAGGAGGAGGCTCAGTCTCTGATAGAGGAG-3' (forward) and 5'-CTCCTCTATCAGAGACTGAGCCTCCTCCTGAAT-3' (reverse), and the plasmid of CYP2B6 WT (pLWCYP2B6dH) were used to amplify the plasmid of the C152S mutant. For the C475S mutant, the mutagenic primers were 5'-CTGACACCCCAGGAGTCTGGTGTGGG-3' (forward) and 5'-CCCACACCAGACTCCTGGGGTGTTCAG-3' (reverse). The entire mutant genes for the C152S and C475S mutants were sequenced in one direction by the Biomedical Sequencing Core Facilities at the University of Michigan to confirm the mutation. Cytochrome P450 reductase, cytochrome *b*₅, CYP2B6, CYP2B6 C152S, CYP2B6 C475S, and CYP2B1 were expressed and purified as described previously.²²⁻²⁴

Determination of the Kinetic Parameters and Partition Ratios for the Mechanism-Based Inactivation of CYP2B6 and CYP2B1 by the Organothiophosphate Pesticides.

The kinetics for the inactivation of CYP2B6 and CYP2B1 by OP pesticides were determined at 37 °C in a reconstituted system. The primary reaction mixtures contained 120 nM CYP2B6 or CYP2B1, 240 nM CPR, 120 nM cytochrome *b*₅, 15 ng/ μ L DLPC, 57 units/mL catalase, and 0 to 6.4 μ M CPS or 0 to 2.4 μ M CPS-methyl, diazinon, azinophos-methyl, or parathion-methyl in 50 mM potassium phosphate buffer at pH 7.4. The reactions were initiated by the addition of NADPH to a final concentration of 0.69 mM. At the designated incubation times, 40 μ L aliquots of the primary reaction solution were transferred to 1000 μ L of a secondary reaction solution that contained 0.1 mM 7-EFC and 0.2 mM NADPH in 50 mM potassium phosphate buffer at pH 7.4. The secondary reaction mixture was then incubated for 5 min at 37 °C before the reaction was terminated by the addition of 330 μ L of ice-cold acetonitrile. The fluorescence of 7-hydroxy-4-trifluoromethylcoumarin was measured with excitation at 410 nm and emission at 510 nm using a Victor II microtiter plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA). The K_I and k_{inact} values were obtained by fitting the rates of inactivation determined at various concentrations of the inactivator to the Michaelis-Menten equation using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

To determine the partition ratios, the primary reaction mixtures were setup as described above and contained molar ratios of CPS to CYP2B6 ranging from 0 to 40.5, and they were incubated at 37 °C for 2, 4, or 6 min after the addition of 1 mM NADPH. The activity remaining after the inactivation of CYP2B6 was determined in a secondary reaction mixture as described above and plotted against the molar ratio of CPS to CYP2B6. The partition ratio was then determined as described previously.²⁵

Analysis of the Protein Adducts of CYP2B6 by ESI-LC/MS.

To examine whether CPS modified the apoprotein of CYP2B6, we determined the molecular mass of the inactivated CYP2B6. CYP2B6 was inactivated at 37 °C by incubation in the primary reaction mixture in the presence of 10 μ M CPS. After 5 min of incubation, aliquots

of 50 μL of the primary reaction mixture were loaded onto a reverse-phase C_3 column and eluted into a LCQ Classic ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) to determine the molecular masses of the inactivated protein as described previously.²⁶ To remove any disulfide bonds or to reverse aggregation, aliquots of 50 μL of the primary reaction mixture that had been inactivated by CPS were incubated with 10 mM DTT at room temperature for 1 h. The molecular mass of the DTT-treated CYP2B6 was then determined by ESI-LC/MS as described above.

Dialysis Experiments for the Determination of the Irreversibility of Inactivation of CYP2B6 by CPS.

The primary reaction mixtures contained 250 nM CYP2B1 or CYP2B6, 500 nM CPR, 250 nM cytochrome b_5 , 30 ng/ μL DLPC, and 5 μM CPS in 50 mM phosphate buffer at pH 7.4. The reactions were initiated by the addition of NADPH to a final concentration of 1 mM or with water for the control reactions. The reaction mixtures were incubated for 1 min before they were placed on ice to stop the reaction. The reactions were then dialyzed against 50 mM potassium phosphate buffer, pH 7.4, in 3 mL Slide-A-Lyzer G2 Dialysis cassettes (Thermo Fisher Scientific). The buffer was exchanged every 15 min over the course of an hour for a total of four buffer exchanges. Following dialysis, a 20 μL aliquot was transferred to a 1 mL secondary reaction solution that contained 0.1 mM 7-EFC and 0.2 mM NADPH in 50 mM potassium phosphate buffer at pH 7.4. The secondary reaction mixture was then incubated for 5 min at 37 °C before it was terminated by the addition of 330 μL of ice-cold acetonitrile. The fluorescence of 7-hydroxy-4-trifluoromethylcoumarin was measured as described above.

Determination of the Kinetic Parameters for the Formation of Chlorpyrifos-Oxon by CYP2B1 and CYP2B6.

The kinetics for the formation of CPO by CYP2B1 and CYP2B6 were determined at 37 °C in a reconstituted system. For CYP2B6, the reaction mixtures contained 16 nM CYP2B6, 32 nM CPR, 16 nM cytochrome b_5 , 2 ng/ μL DLPC, 8 units/mL catalase, 0 to 8 μM CPS, and 50 mM potassium phosphate buffer, pH 7.4, in a final volume of 125 μL . For CYP2B1, the reaction mixtures contained 40 nM CYP2B1, 80 nM CPR, 40 nM cytochrome b_5 , 5 ng/ μL DLPC, 80 units/mL catalase, 0 to 3.2 μM chlorpyrifos, and 50 mM potassium phosphate buffer, pH 7.4, in a final volume of 150 μL . The reactions were initiated by the addition of NADPH to a final concentration of 0.4 (CYP2B6) or 0.8 mM (CYP2B1). Reaction mixtures were incubated for 10–15 s prior to stopping with an equal volume of ice-cold acetonitrile and the addition of 5 (CYP2B1) or 6.4 nM (CYP2B6) CPS-methyl oxon as an internal standard. The samples were centrifuged, and 50- μL aliquots of the supernatant were analyzed using an LC/UV/MS system, which consisted of a Waters 2690 Separations Module (Milford, MA) and a triple-quadrupole mass spectrometer. Chromatographic separations were achieved on a Phenomenex SynergiHydro-RP column (4 μm , 2.0 $\times$ 50 mm) heated to 35 °C using mobile phase A (water with 0.1% acetic acid) and mobile phase B (acetonitrile with 0.1% acetic acid). The HPLC elution gradient started with 35% B followed by an increase to 40% B over 2 min directly followed by another increase to 95% B over 2 min. The column was then washed with 95% B for 2.3 min before returning to the initial conditions. The column was equilibrated for 4.6 min between injections. The flow rate was

0.35 mL/min, and all gradients were linear. The HPLC column effluent was analyzed using a Thermo Fisher TSQ Quantum Ultra triple-quadrupole mass spectrometer equipped with an electrospray ionization source. The mass spectrometer was operated in positive mode with nitrogen as both the sheath gas and auxiliary gas. It was adjusted so as to obtain the maximum sensitivity based on the parent molecule. The source temperature was set to 350 °C, the electron spray voltage was 3.4 kV, and the normalized collision energy was at 17–19 eV. All samples were analyzed with single reaction monitoring mass acquisition using the following transitions: 349.8–277.8 m/z for CPS, 333.8–277.8 m/z for CPO, and 305.7–273.7 m/z for CPS-methyl oxon. Data were processed using Xcalibur software (Thermo Fisher Scientific). Peak area ratios of authentic standards to the internal standard were used to generate a standard curve permitting calculation of the amount of CPO produced. Nonlinear regression and enzyme kinetic analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA).

IC₅₀ Shift Experiments.

IC₅₀ shift experiments with a preincubation step in the presence or absence of NADPH were conducted at 37 °C in a reconstituted system. The primary reaction mixtures contained 0.5 μ M CYP2B1, CYP2B6, CYP2B6 C152S, or CYP2B6 C475S, plus 1 μ M CPR, 0.5 μ M cytochrome *b*₅, 60 ng/ μ L DLPC, 0.5 unit/ μ L catalase, and 0 to 3.2 μ M CPS in 50 mM phosphate buffer at pH 7.4. The reactions were initiated by the addition of NADPH to a final concentration of 1.2 mM or with water for the controls. The reaction mixtures were incubated for 3 or 10 min before a 12 μ L aliquot of the primary mixture was transferred to a 300 μ L secondary reaction solution that contained 0.1 mM 7-EFC and 0.3 mM NADPH in 50 mM potassium phosphate buffer at pH 7.4. The secondary reaction mixture was then incubated for 10 min at 37 °C before it was terminated by the addition of 50 μ L of ice-cold acetonitrile. The fluorescence of 7-hydroxy-4-trifluoromethylcoumarin was measured as described above. The IC₅₀ values were obtained by nonlinear regression using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

Loss of Heme Content As Measured by High-Performance Liquid Chromatography and the Spectrum of the Carbon Monoxide Complex of the Ferrous P450.

To examine whether mechanism-based inactivation of CYP2B6 by CPS was due to modification of the heme, we measured the native heme remaining and the ferrous CO-P450 spectrum after the inactivation reaction. For measurement of the native heme, the reaction mixtures contained 1 μ M CYP2B6, 2 μ M CPR, 1 μ M cytochrome *b*₅, 0.12 μ g/ μ L DLPC, and 0 or 100 μ M CPS in 50 mM phosphate buffer at pH 7.4. The inactivation reaction was initiated by the addition of NADPH to give a final concentration of 1 mM. The reactions were stopped after 0, 15, 30, 45, or 60 s by placing them on ice. A 50 μ L aliquot was injected onto an HPLC equipped with a UV detector as described previously.²⁷ For the measurement of the ferrous CO-P450 spectrum, the reaction mixtures contained 0.4 μ M CYP2B6, 0.8 μ M CPR, 0.4 μ M cytochrome *b*₅, 0.05 μ g/ μ L DLPC, 0.1 unit/ μ L catalase, and 100 μ M CPS in 50 mM phosphate buffer at pH 7.4. The inactivation reaction was initiated by the addition of NADPH to a final concentration of 0.2 mM. The reactions were stopped by the addition of a few grains of dithionite after 0, 15, 30, 45, or 60 s and then bubbled with

CO. The UV–visible spectra of the ferrous CO-P450 complexes were recorded from 400 to 500 nm on a Shimadzu UV/vis spectrophotometer (UV-2501PC; Shimadzu, Kyoto, Japan).

Rate of Electron Transfer from CPR to the Ferric CYP2B6.

The rate of electron transfer from CPR to the ferric CYP2B6 was determined at 25 °C using an SF61DX2 stopped-flow spectrophotometer (TgK Scientific, Bradford-on-Avon, UK) as described previously.²⁶ To preform the P450–CPR complex, equimolar amounts of CYP2B6 and CPR (6 nmoles each) were incubated on ice for 1 h with 0.15 $\mu\text{g}/\mu\text{L}$ DLPC. The preformed complex was then added to give a final concentration of 3 μM in 0.1 M potassium phosphate buffer, pH 7.4, containing either 0 or 10 μM CPS. The preformed complex was rapidly mixed in the stopped-flow spectrophotometer with an equal volume of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM NADPH. Both solutions had been saturated with carbon monoxide prior to mixing. The kinetics of electron transfer were monitored at 450 nm. The rate constants were obtained by fitting the kinetic traces at 450 nm to exponential functions using KinetAsyst software (TgK Scientific).

SDS–PAGE Analysis of the Inactivated CYP2B6.

Primary reaction mixtures contained 4.3 μM CYP2B6, 8.6 μM CPR, 4.3 μM cytochrome *b5*, and 100 μM CPS in 50 mM phosphate buffer at pH 7.4. The reactions were initiated by the addition of NADPH to a final concentration of 0.6 mM or with water for the control reactions. Both reactions were incubated for 10 min before they were placed on ice to stop the reaction. Following the inactivation, 170 pmol of P450 was mixed with Laemmli sample buffer containing either 0 or 3.92 mM β -mercaptoethanol, heated for 5 min in a boiling water bath, and loaded onto a 10% polyacrylamide gel and separated by SDS–PAGE (Bio-Rad minigel). After running, the gel was stained with Coomassie blue for visualization of the proteins. Dual color Precision Plus Protein standards (Bio-Rad, Hercules, CA) were also run on the gels as molecular weight markers.

RESULTS

Mechanism-Based Inactivation of CYP2B6 by Chlorpyrifos and Other Structurally Related Organophosphate Pesticides.

The kinetic parameters for the mechanism-based inactivation of CYP2B6 were determined in the reconstituted system. Incubation of CYP2B6 in the presence of CPS led to the loss of 7-EFC *O*-deethylase activity in a time- and concentration-dependent manner (Figure 1). The K_I was determined to be 0.47 μM , and the k_{inact} was determined to be 1.97 min^{-1} ($t_{1/2}$ = 0.35 min), resulting in an inactivation efficiency (k_{inact}/K_I) of 4.2 (Table 1). The addition of GSH to the primary reaction mixtures did not protect CYP2B6 from being inactivated by CPS, and the loss of activity could not be recovered following overnight dialysis (data not shown).

In order to determine if the inactivation of CYP2B6 was unique to CPS, we tested the structurally related organo-phosphate pesticides CPS-methyl, parathion-methyl, azinophos-methyl, and diazinon (Figure 2). Potent inactivation of CYP2B6 was observed with all the organophosphate pesticides tested (Table 1). CPS had the fastest rate of inactivation (1.97

min^{-1}) while parathion-methyl displayed the lowest K_I ($0.19 \mu\text{M}$) and highest k_{inact}/K_I (6.42).

Partition Ratio for the Mechanism-Based Inactivation of CYP2B6 by Chlorpyrifos.

The partition ratio for the mechanism-based inactivation of CYP2B6 by CPS was determined in the reconstituted system. The 7-EFC *O*-deethylase activity remaining for the inactivated CYP2B6 decreased with increasing molar ratios of CPS to CYP2B6, as expected (Figure 3). The partition ratio was measured under conditions where the inactivation was allowed to continue until completion (2 min), and the activity remaining was approximately 2% of that of the control at the highest molar ratio of CPS to CYP2B6. The partition ratio was determined to be 17.7, suggesting that, on average, multiple rounds of metabolism occurred prior to an inactivation event.

Kinetic Parameters for the Formation of Chlorpyrifos Oxon by CYP2B6.

Previous studies suggested that the release of a sulfur molecule during the metabolism of parathion to parathion oxon was the mechanism for the inactivation of P450s.¹⁶ On the basis of the rapid rate of inactivation and the partition ratio observed for the inactivation of CYP2B6 by CPS, we reasoned that the metabolism of CPS to CPO must be very rapid in the reconstituted system if inactivation occurred through the previously proposed mechanism. Thus, we measured the kinetics of desulfuration of CPS by CYP2B6. The kinetic parameters were determined at concentrations ranging from 0 to $8 \mu\text{M}$ CPS. Initial experiments indicated that the rate of CPO formation began to drift away from linearity as early as 10 s, an observation consistent with the observed loss of 7-EFC *O*-deethylase activity at 15 s during the analysis of mechanism-based inactivation. We chose 10 s incubation times as a compromise between accurate measurement and loss of activity to estimate the kinetic parameters. The apparent K_m , k_{cat} , and catalytic efficiency (k_{cat}/K_m) parameters were $0.22 \pm 0.06 \mu\text{M}$, $9.34 \pm 1.74 \text{ min}^{-1}$, and $42.7 \mu\text{M}^{-1} \text{ min}^{-1}$, respectively (Figure 4), and are in good agreement with previous results.¹⁵ The k_{cat} value, which is likely underestimated due to inactivation occurring during the incubations, indicates a rapid turnover of CPS to CPO.

To further investigate the rapid turnover of CPS to CPO during the reaction, we measured the rate of electron transfer from CPR to CYP2B6, a critical step that must occur prior to substrate hydroxylation in the catalytic cycle, in the presence and absence of CPS. The kinetic traces were both best fit with double exponential equations to give the apparent rate constant, k_{obs} values (Table 2). The k_{obs} for the fast phase of the electron transfer to CYP2B6 (k_1) in the absence of a substrate was 0.12 s^{-1} , a value which is similar to the value of 0.31 s^{-1} calculated previously for CYP2B6.²⁸ In contrast, the k_1 in the presence of CPS was 2.1 s^{-1} , which is approximately 17.5-fold faster. A similar magnitude of increase (~20-fold) in k_1 was observed for CYP2B4 in the presence of benzphetamine when compared to that in the absence of benzphetamine,²⁶ which is a substrate of CYP2B4 that is rapidly turned over with a k_{cat} value of 32 min^{-1} .²⁹

Potential Aggregation of CYP2B6 during Mechanism-Based Inactivation by Chlorpyrifos.

Previous studies of rat liver P450s during the metabolism of parathion indicated the formation of high-molecular weight aggregates which were detected by SDS-PAGE

analysis.¹⁶ We conducted SDS–PAGE analysis to see if CYP2B6 was forming similar high-molecular weight aggregates following inactivation. When CYP2B6 was incubated with CPS in the absence of NADPH and then run on the gel, two large bands representing CPR (74 000 Da) and CYP2B6 (54 400 Da), respectively, were seen near the 75 and 50 kDa molecular weight markers (Figure 5, lane 1). The bands were very wide and ran slightly faster than the expected molecular weights, likely due to the large amounts of protein loaded on the gel. When the sample incubated with NADPH was run on the gel, the bands representing CYP2B6 and CPR exhibited decreases in intensity (Figure 5, lane 2). In addition, a shadow extending from the origin to the band for CPR with a darker area above the 200 kDa molecular weight marker can be observed, which was not present in the no NADPH sample. The decrease in intensity of the CYP2B6 band and the additional dark areas above 200 kDa were suggestive that inactivation of CYP2B6 by CPS leads to the formation of some high-molecular-weight aggregates of CYP2B6. When 2-mercaptoethanol was added to the NADPH sample prior to running on the gel, the dark area above 200 kDa disappeared, and a significant increase in the intensity of the CYP2B6 band was observed (Figure 5, lane 4). This finding supports that, as was observed previously with parathion, the aggregates are formed through disulfide linkages.

Analysis of the Molecular Mass of the CYP2B6 Protein Inactivated by Chlorpyrifos.

To examine whether the inactivation of CYP2B6 by CPS led to the formation of protein adducts, we attempted to determine the molecular mass of the inactivated CYP2B6 by ESI-LC/MS. In the absence of NADPH, CYP2B6 exhibited a molecular mass of 54, 419 Da (Figure 6), which corresponds to the expected mass for unmodified CYP2B6 and is very similar to that determined previously.²² However, when the inactivated protein was analyzed by ESI-LC/MS, no peak was detected by the mass spectrometer. We reasoned that this may be due to the formation of high molecular weight aggregates, as was observed previously¹⁶ and was also observed during our SDS–PAGE analysis, that may have interfered with the ability of the proteins to elute from the HPLC column and enter the MS detector or that electrospray ionization of the aggregated proteins inside the MS source was hindered, thereby preventing detection. Therefore, we treated the inactivated protein with 10 mM DTT prior to ESI-LC/MS, which resulted in some recovery of the peak (Figure 6). This provides additional evidence that disulfide bond formation occurs during the inactivation by CPS and that this modification at least partially interferes with the SDS–PAGE and ESI-LC/MS analyses.

Loss of Heme during the Mechanism-Based Inactivation of CYP2B6 by Chlorpyrifos.

Previous experiments with parathion indicated that loss of native heme and CO-detectable heme occurred during mechanism-based inactivation of P450.¹⁶ Thus, we investigated the loss of heme during the mechanism-based inactivation of CYP2B6 by CPS. Over the course of 45 s, CYP2B6 lost approximately 80% of the 7-EFC *O*-deethylase activity and 87% of the CO-detectable heme (Table 3 and Figure 7). In addition, an increase in a Soret band at 420 nm was observed over the course of the experiment, indicating modified heme (Figure 7). In contrast to the loss of CO-detectable heme, only 45% of the native heme as measured by HPLC was lost after 15 s and no further significant loss was observed throughout the experiment (data not shown). However, the experiment was conducted in the presence of

cytochrome *b*₅, a heme containing protein, at a ratio of 1:1 compared to CYP2B6. Thus, the remaining native heme detected in the experiment is likely that of cytochrome *b*₅ and not CYP2B6. Therefore, we repeated the experiment in the absence of cytochrome *b*₅. In the absence of cytochrome *b*₅, the mechanism-based inactivation was much slower; CYP2B6 lost 65% of the 7-EFC *O*-deethylase activity after 5 min (Table 4). CYP2B6 also lost 67% of the native heme after 5 min. In light of the possibility that the previously observed protein aggregation could confound the results by preventing the intact heme from dissociating from the proteins, we repeated the experiments with the addition of 2 mM DTT prior to analysis by HPLC. The addition of DTT did not alter the results (Table 4), suggesting that the decreased levels of native heme measured represent true loss of heme and not aggregation.

Lack of Mechanism-Based Inactivation during the Metabolism of CPS by CYP2B1.

We investigated the inactivation of CYP2B1, the rat homologue of CYP2B6, for two major reasons: (1) animal models, such as rat, will be useful in investigating whether the inactivation of members of the CYP2B family by CPS is relevant in an in vivo system, and (2) CYP2B1 has Cys residues at the same positions as CYP2B6 except at position 475, which is the key Cys residue thought to be involved in inactivation by 2-oxo-clopidogrel.²² Inactivation was evaluated by comparing the IC₅₀ values following a preincubation with or without NADPH. CPS incubated with CYP2B6 exhibited a lower IC₅₀ value (0.15 μ M) when NADPH was added to the preincubation step compared to the no NADPH control (0.27 μ M), consistent with our previous experiments demonstrating that CPS is a mechanism-based inactivator of CYP2B6 (Figure 8A). In contrast, there was essentially no difference in IC₅₀ values between the NADPH and no NADPH preincubations when CYP2B1 was incubated with CPS (Figure 8B), even when the preincubation time was increased from 1 to 10 min (data not shown). The lack of inactivation of CYP2B1 by CPS was confirmed using the time- and concentration-dependent mechanism-based inactivation assay described earlier for CYP2B6. CPS appeared to inhibit CYP2B1 in a concentration-dependent manner only, suggesting the inhibition was competitive and not due to mechanism-based inactivation (Figure 9B). In order to rule out the possibility that CYP2B1 would not be inactivated since it was unable to produce CPO and therefore release the activated sulfur which is proposed to be involved in inactivation, we performed preliminary experiments to measure the formation of CPO by CYP2B1. CYP2B1 formed a significant amount of CPO at similar concentrations (0–3.2 μ M) to those used in the MBI experiments, and the initial rates measured were somewhat faster than those measured previously for CYP2B6 (data not shown).

Effect of the C475S Mutation on the Mechanism-Based Inactivation of CYP2B6 by CPS.

CYP2B6 has five Cys residues, four of which are also found in the same position in CYP2B1. The Cys located at position 475 is absent in CYP2B1. We postulated that C475 may therefore be the critical site of modification on the apoprotein that could be modified by sulfur and would result in inactivation of CYP2B6 during the metabolism of CPS. To test this, we measured the ability of CPS to inactivate two variant CYP2B6 proteins created by site-directed mutagenesis in which two of the Cys residues were replaced with Ser residues, CYP2B6 C475S, and CYP2B6 C152S. Inactivation was evaluated by comparing the IC₅₀ values following a preincubation with or without NADPH. On the basis of the levels of

activity for the metabolism of 7-EFC in the absence of CPS, both mutants had similar basal activity which was comparable to the basal activity for WT CYP2B6 under the same conditions. As expected, CPS incubated with CYP2B6 C152S exhibited a lower IC_{50} value ($0.19 \mu M$) when NADPH was added to the preincubation step compared to the no NADPH control ($0.38 \mu M$), suggesting that C152 does not play a major role in the inactivation of CYP2B6 by CPS (data not shown). A similar result was observed for CPS incubated with CYP2B6 C475S (data not shown), and a lower IC_{50} value ($0.14 \mu M$) was observed when NADPH was added to the preincubation step compared to the no NADPH control ($0.24 \mu M$). This suggests that C475 also does not play a major role in the inactivation of CYP2B6 by CPS. The results of the IC_{50} experiments were confirmed using the time- and concentration-dependent mechanism-based inactivation assay described earlier for CYP2B6. Incubations with CPS led to clear time- and concentration-dependent inhibition of both CYP2B6 C152S and CYP2B6 C475S (Figures 9 C and D). The estimated kinetic parameters were K_I values of 0.83 and $1.29 \mu M$ and k_{inact} values of 2.51 and 2.38 min^{-1} for CYP2B6 C152S and CYP2B6 C475S, respectively, giving k_{inact}/K_I values of 3.02 (C152S) and 1.84 (C 475S).

DISCUSSION

In this study, we investigated the mechanism-based inactivation of CYP2B6 by CPS in a reconstituted system and determined the kinetics for the inactivation. Our results indicate that CPS is an extremely potent mechanism-based inactivator of CYP2B6. To our knowledge, CPS is a more potent mechanism-based inactivator of CYP2B6 than any other chemical previously identified based on the efficiency of inactivation. For example, CPS is even more efficient than clopidogrel,^{22,30} a clinically used drug known to result in CYP2B6 drug–drug interactions.³¹ A number of other OP pesticides tested were similarly potent inactivators of CYP2B6, including parathionmethyl which was even more efficient than CPS (Table 1). Since a number of OPs in this study were more efficient than clopidogrel, and clopidogrel is known to interfere with the metabolism of other drugs by CYP2B6, there is concern that exposure to OPs could result in altered drug metabolism or sensitivity to environmental chemicals which are cleared by CYP2B6, including further exposure to the OPs themselves. Further studies are needed to confirm that inactivation can occur in humans and what the resulting consequences on drug/xenobiotic metabolism would be.

On the basis of previous experiments, the inactivation of CYP2B6 by CPS is most likely to be related to the release of sulfur following oxidative desulfuration.¹⁶ Our results for the rate of CPO formation, the metabolite of CPS following oxidative desulfuration, by CYP2B6 were lower than expected based on the kinetics of inactivation. Since the partition ratio is a ratio of the k_{cat} to the k_{inact} , we expected a k_{cat} value of 34.9 min^{-1} for the formation of CPO from CPS by CYP2B6. However, the calculated value in our study was significantly smaller at 9.4 min^{-1} , and a similar k_{cat} was reported previously for CYP2B6.¹⁵ It should be pointed out that the value calculated in our results is likely an underestimation of the true k_{cat} because it was measured under conditions where inactivation was occurring. Although the inactivation of CYP2B6 was not investigated in the previous study, it is likely that inactivation was occurring during the 2 min incubation times used. Additional data generated in our study suggests that CYP2B6 could support a rate of metabolism closer to the expected rate. The first was that the rate of electron transfer from CYP2B6 to CPR was

enhanced by nearly 20-fold in the presence of CPS (Table 2). This value is similar to the value observed for the stimulation of CYP2B4 reduction in the presence of benzphetamine,²⁶ a substrate that CYP2B4 turns over with a k_{cat} of 32 min^{-1} .²⁹ The second was that CYP2B1, which was not inactivated by CPS, had a k of $34.5 \text{ min}^{-1} \text{ cat}$ for the formation of CPO, indicating that a structurally similar CYP2B can support a high rate of turnover.

Under our experimental conditions, CPS was only a competitive inhibitor of CYP2B1 and did not cause inactivation. This finding was unexpected since it has been shown previously that parathion leads to mechanism-based inactivation of CYP2B1.¹⁶ Another group investigated the inhibition of CYP2B1 by parathion and found that competitive inhibition was observed at low concentrations and that inactivation only occurred at concentrations of $5 \mu\text{M}$ or greater.^{32,33} In the experiments described by Neal and Halpert, relatively high concentrations of parathion ($50 \mu\text{M}$) were used; a concentration where Murray and Butler³² also observed inactivation. Thus, it is possible that CPS can inactivate CYP2B1 at higher concentrations. However, we were unable to determine if CYP2B1 would be inactivated with higher concentrations of CPS because of the potent competitive inhibition of 7-EFC *O*-deethylase activity in the secondary reaction and the low water solubility of CPS. It is important to keep in mind that, except for accidental exposures to large amounts of CPS or intentional poisonings, environmental exposure to OPs are likely to be relatively low, and therefore, lower concentrations of CPS are more relevant for risk assessment. On the basis of our findings here that CYP2B1 is not inactivated at low concentrations of CPS, animal models may not be useful for predicting the potential effects of inactivation caused by OPs to humans for CYP2B substrates.

It is unclear why CYP2B1 is not inactivated by low concentrations of parathion or CPS. Both compounds are rapidly converted to the oxon form which suggests that sulfur release is occurring at concentrations where inactivation is not observed. Murray and Butler³² suggested that parathion may out-compete the substrate for binding but have a lower k_{cat} than the competing substrate. This does not seem to be the case for CYP2B1 and CPS as the k_{cat} for CPO formation is greater than that observed for 7-EFC.³⁴ It is likely that small structural differences between CYP2B6 and CYP2B1 contribute to the different susceptibilities for inactivation by CPS.

In previous experiments with parathion, it was observed that loss of enzymatic activity was greater than the loss of spectrally detectable P450 as its carbon monoxide complex, and the loss of P450 was greater than the loss of heme suggesting that neither finding could completely explain the loss of activity.¹⁶ In contrast, our experiments showed that in the absence of cytochrome *b*₅ the loss of detectable heme was approximately equivalent to the observed loss of activity (Table 4), and in experiments with cytochrome *b*₅, we observed that the loss of detectable P450 was approximately equivalent to the observed loss of activity (Table 3). It is difficult to discern the exact reasons for the differences observed between our work and that of Halpert and Neal because of the use of different enzymes, pesticides and experimental conditions. We were concerned that the potential aggregation of CYP2B6, suggested by our SDS-PAGE and ESI-LC/MS experiments following inactivation may have prevented the release of heme and thus would appear as heme loss in our experiment. However, treatment with DTT following inactivation, which greatly reduced the level of

aggregation observed (Figure 5) and partially restored the ability to detect CYP2B6 by ESI-LC/MS, was not able to prevent any of the observed loss of detectable heme. Therefore, under our experimental conditions it appears that loss of detectable heme is the major mechanism leading to inactivation of CYP2B6 by CPS, as opposed to previous results with animal P450s where heme loss only accounted for 50% of the loss of activity.¹⁶

The exact nature of the mechanism leading to the observed heme loss is unclear. It was previously proposed that loss of heme may be caused by sulfur directly attacking the heme followed by dissociation of the heme iron from the porphyrin ring.³⁵ In line with this idea, computational experiments support the formation of an inhibitory complex with sulfur binding to both the heme iron and nitrogen of the porphyrin ring.³⁶ We measured heme loss by looking at the total amount of native heme released from CYP2B6. We considered the possibility that the sulfur adducted heme might leave the inactivated CYP2B6 and therefore be able to be detected by our assay, but no adducted heme was observed in our experiments. However, it is also possible that the sulfur adducted heme may have been unstable or it may absorb at a different wavelength than native heme, resulting in the decreased detection of heme observed. Another possibility is that the heme was damaged but did not dissociate from the CYP2B6 following inactivation. It has previously been shown that at least part of the heme was covalently bound to CYP2B1 following metabolism by parathion.³⁷ It should also be pointed out that we did observe a small amount of absorbance at 420 nm during the analysis of the reduced CO-P450 complex which indicated at least some of the inactivated proteins still had altered heme present in the active site.

The majority of the sulfur released during the metabolism of parathion binds to Cys residues and is thought to be involved in the inactivation.¹⁶ Our results presented here support the suggestion that sulfur binding to Cys residues of CYP2B6 contributes to some type of alteration of the protein which is at least partially reversible by the addition of reducing agents. CYP2B6 contains five Cys residues, four of which are located in the same position in CYP2B1. Since CYP2B1 was not inactivated, despite efficient metabolism of CPS with release of sulfur, we reasoned that the four Cys residues shared by CYP2B1 and CYP2B6 do not play a major role in inactivation. To support this, mutation of C152 in CYP2B6, which is conserved in CYP2B1, to Ser did not protect against inactivation. Interestingly, mutation of C475, which is present in CYP2B6 but not CYP2B1, also did not protect against inactivation. Furthermore, treatment of inactivated CYP2B6 with DTT was unable to reverse the observable loss of heme or activity even though it reversed aggregation. Similar experiments with parathion and P450s showed that treatment with agents that disrupt disulfide bonds were not able to restore activity.¹⁶ Taken together, it appears that binding of sulfur to Cys residues in CYP2B6 does not play a major role in inactivation by CPS.

The potent mechanism-based inactivation of CYP2B6 by CPS and other OP pesticides has the potential to result in OP–drug or OP–xenobiotic interactions in humans. While this may not be of concern for the majority of the population where OP exposure is expected to be low, the USEPA has identified certain subpopulations where exposure levels may be of concern, such as residents near OP pesticide application sites and occupational workers.^{8,9} A pharmacokinetic study conducted in human volunteers showed that the concentration of the major metabolite of CPS, TCP, reached a maximum value of 0.063 $\mu\text{g/mL}$ (0.32 μM) in

blood following a 5.0 mg/kg dermal dose of CPS.³⁸ According to estimates by the USEPA, occupational exposures in certain scenarios may result in daily dermal doses that are equal to or greater than 5.0 mg/kg/day.³⁹ The blood concentration of TCP is the result of significant first pass metabolism, and therefore, the concentration of CPS that reached the liver is expected to be similar or even higher. The K_I values for the inactivation of CYP2B6 by OP pesticides (0.19–0.80 μM) are all similar to the measured concentration of TCP suggesting that inactivation is likely to be occurring under physiologically relevant concentrations following occupational exposures.

In addition to interactions with other compounds, inactivation of CYP2B6 by OP may alter the risk of toxicity of a subsequent OP exposure since CYP2B6 is one of the major human P450s involved in CPS metabolism.¹⁴ This is particularly critical since repeated exposure would be expected for occupational workers who are also the population which is expected to have the highest exposure and therefore are the population at highest risk. It is believed that due to the rapid metabolism of CPO in blood to the nontoxic TCP, that local bioactivation of CPS to CPO in the brain is the most likely mechanism for acetylcholinesterase inhibition.^{6,21,40} Therefore, if CPS or other OPs inactivate CYP2B6, there may be less clearance of CPS in a second exposure. This may result in higher levels of CPS reaching the brain and increase the potential for toxicity. This is supported by data in rats where intraperitoneal administration of an MBI of CYP2B1 resulted in increased levels of CPS in the serum and brain due to a lack of bioactivation to CPO.⁴⁰ While the CYP2B1 inhibitor was also able to block brain ChE inhibition by preventing the formation of CPO in the brain, this may not be the case for repeat exposure to OPs in humans. For example, it is possible that some level of initial exposure to CPS may be enough to result in significant inhibition of CYP2B6 in the liver yet the resulting levels of CPS that reach the brain do not result in significant bioactivation, and therefore, relatively little MBI of CYP2B6 in the brain would occur. In a subsequent exposure, more CPS would reach the brain due to the inactive CYP2B6 in the liver which would then be bioactivated in the brain because the brain CYP2B6 would still be active. Human physiologically based pharmacokinetic/pharmacodynamic models of CPS metabolism are currently available to help predict human metabolism of CPS.⁴¹ However, these models currently do not take into account multiple dosing and the impact of mechanism-based inactivation. On the basis of the potent inactivation observed in this study, we feel that in order to better predict CPS toxicity, the potential impact of inactivation on the pharmacokinetics of CPS in humans should be considered.

In summary, we have demonstrated that CPS and other OP pesticides lead to potent mechanism-based inactivation of CYP2B6 and that the inactivation observed is more potent than that observed with other CYP2B6 MBIs. The potent inactivation observed in our study supports the need for further investigations into potential interactions of OP pesticides with drugs or xenobiotics metabolized by human CYP2B6 *in vivo*. In addition, further characterization of the inactivation of other human P450s by OP pesticides, such as CYP3A4 which is also inactivated by CPS,¹⁹ is needed to predict the full potential of OP pesticides to lead to altered metabolism of drugs and xenobiotics. We also investigated the mechanism of inactivation of CYP2B6 by CPS. A number of our mechanism studies were similar to those previously conducted with animal P450s and support that a similar

mechanism is involved in the inactivation of human CYP2B6 by CPS, although a few subtle differences were observed. For example, our data support the conclusion that heme loss was the major mechanism leading to the inactivation of CYP2B6 as opposed to only accounting for ~50% of the loss of activity in previous experiments with animal P450s.¹⁶

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ABBREVIATIONS

CPO	chlorpyrifos-oxon
CPS	chlorpyrifos
DLPC	L- α -dilauroylphosphatidylcholine
DTT	dithiothreitol
7-EFC	7-ethoxy-4-trifluoromethylcoumarin
GSH	glutathione
MBI	mechanism-based inactivator
OP	organothiophosphate
TCP	3,5,6-trichloro-2-pyridinol

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^aA represents the desulfuration pathway and B the dearylation pathway. S represents sulfur released during desulfuration.

Scheme 1.

Proposed Pathways for the Metabolism of Chlorpyrifos by Cytochrome P450s^a

Figure 1.

Kinetics for the mechanism-based inactivation of CYP2B6 by chlorpyrifos. The inactivation reactions were performed at 37 °C in the reconstituted system as described in Materials and Methods. (A) The concentrations of chlorpyrifos in the primary reaction were 0 (red circle), 0.4 (orange square), 0.8 (yellow triangle), 1.6 (green triangle), 3.2 (blue diamond), and 6.4 μM (purple star). (B) The plot of the observed rates (k_{obs}) at various concentrations of chlorpyrifos, which were used to calculate the kinetic parameters K_{I} and k_{inact} . The results are the average $\pm$ SD of four experiments conducted under the same conditions.

Figure 2.
Chemical structures of the organothiophosphate pesticides used in this study.

Figure 3.

Determination of the partition ratio for the inactivation of CYP2B6 by chlorpyrifos. The percentage of catalytic activity remaining was determined as a function of the molar ratio of chlorpyrifos to CYP2B6 as described in Materials and Methods. The partition ratio was estimated from the intercept of the linear regression lines for the high and low ratios of chlorpyrifos to CYP2B6. The data presented here are representative of experiments conducted at multiple time points giving similar results.

Figure 4.

Michaelis–Menten plot for the formation of chlorpyrifos oxon by CYP2B6. CYP2B6 was incubated with varying concentrations of chlorpyrifos at 37 °C for 10 s, and the formation of chlorpyrifos oxon was measured as described in Materials and Methods. The rate of the reaction at each concentration (pmol chlorpyrifos oxon/min/pmol of P450) was plotted against the concentration of chlorpyrifos and used to calculate the k_{cat} and K_{m} for the formation of chlorpyrifos oxon. The results are the average $\pm$ SD of four experiments conducted under the same conditions.

Figure 5.

SDS-PAGE analysis of CYP2B6 after inactivation by chlorpyrifos. CYP2B6 was inactivated at 37 °C for 10 min in the presence of 100 μ M chlorpyrifos as described in Materials and Methods. Following inactivation, the proteins were loaded onto a 10% polyacrylamide gel and separated by SDS-PAGE and stained with Coomassie blue for visualization. Lane 1, CYP2B6 minus NADPH. Lane 2, CYP2B6 plus NADPH. Lane 3, CYP2B6 – NADPH + 3.92 mM β -mercaptoethanol. Lane 4, CYP2B6 + NADPH + 3.92 mM β -mercaptoethanol.

Figure 6.

Analysis of the protein masses of CYP2B6 by ESI-LC/MS after inactivation by chlorpyrifos. CYP2B6 was inactivated at 37 °C for 5 min in the presence of 10 μ M chlorpyrifos as described in Materials and Methods. The solid line represents CYP2B6 which was incubated with chlorpyrifos in the absence of NADPH. The dotted line represents CYP2B6 incubated with chlorpyrifos in the presence of NADPH. The dashed line represents CYP2B6 incubated with chlorpyrifos in the presence of NADPH and treated with 10 mM DTT for 60 min at room temperature prior to analysis.

Figure 7.

Reduced CO difference spectra of reconstituted CYP2B6 incubated with 10 μ M chlorpyrifos. CYP2B6 was incubated with 10 μ M chlorpyrifos at 37 °C for 0 (blue), 15 (yellow), 30 (orange), or 45 (red) seconds and the spectra of the proteins were determined as described in Materials and Methods. The results presented are the average of two experiments with duplicate measurements at each time point.

Figure 8.

Shifted IC_{50} curves determined by preincubation with and without NADPH for incubations of chlorpyrifos with CYP2B6 and CYP2B1. Chlorpyrifos was preincubated with CYP2B proteins at 37 °C with (red circle) and without (blue triangle) NADPH as described in Materials and Methods. The IC_{50} values were obtained by nonlinear regression and are presented in the text. (A) CYP2B6. (B) CYP2B1. The results presented are the average $\pm$ SD of three experiments.

Figure 9.

Kinetics for the mechanism-based inactivation of CYP2B1, CYP2B6 C475S, and CYP2B6 C152S by chlorpyrifos. The inactivation reactions were performed as described in Materials and Methods. The results presented in B through D are the average of two experiments. (A) The kinetics for CYP2B6 are presented for comparison purposes and are the same as those shown in Figure 1. The concentrations of chlorpyrifos in the primary reaction were 0 (red circle), 0.4 (orange square), 0.8 (yellow triangle), 1.6 (green triangle), 3.2 (blue diamond), and 6.4 (purple star). (B) CYP2B1. The concentrations of chlorpyrifos in the primary reaction were 0 (red circle), 0.2 (orange square), 0.4 (yellow triangle), 0.8 (green triangle), 1.6 (blue diamond), and 3.2 (purple star). (C) CYP2B6 C152S. The concentrations of chlorpyrifos in the primary reaction were 0 (red circle), 0.2 (orange square), 0.4 (yellow triangle), 0.8 (green triangle), 1.6 (blue diamond), and 3.2 (purple star). (D) CYP2B6 C475S. The concentrations of chlorpyrifos in the primary reaction were 0 (red circle), 0.2 (orange square), 0.4 (yellow triangle), 0.8 (green triangle), 1.6 (blue diamond), and 3.2 (purple star).

Table 1.Kinetic Parameters for the Mechanism-Based Inactivation of CYP2B6 by Organophosphate Pesticides^a

pesticide	k_{inact} (min ⁻¹)	K_{I} (μM)	$k_{\text{inact}}/K_{\text{I}}$
chlorpyrifos ($n = 4$)	1.97 ± 0.20	0.47 ± 0.20	4.19
chlorpyrifos-methyl ($n = 3$)	1.58 ± 0.27	0.75 ± 0.28	2.11
parathion-methyl ($n = 3$)	1.22 ± 0.10	0.19 ± 0.06	6.42
diazinon ($n = 3$)	1.31 ± 0.29	0.80 ± 0.14	1.64
azinophos-methyl ($n = 3$)	0.87 ± 0.32	0.31 ± 0.17	2.81

^aThe details for the measurements of the kinetic parameters are given in Materials and Methods. Values represent the means $\pm$ SD from three or four separate experiments.

Table 2.

Kinetic Parameters for the Rate of Electron Transfer from CPR to Ferric CYP2B6 in the Presence and Absence of Chlorpyrifos^a

	kinetic parameters			
	A_1 (%)	k_1 (s ⁻¹)	A_2 (%)	k_2 (s ⁻¹)
CYP2B6 – CPS	49	0.12	51	0.05
CYP2B6 + CPS	65	2.1	35	0.02

^aDetails for the measurements of the kinetic parameters are given in Materials and Methods. k_1 and k_2 are rate constants for the fast and slow phases, respectively, and A_1 and A_2 are their respective relative amplitudes for the fast and slow phases, respectively.

Table 3.

Summary of 7-EFC O-Deethylase Activities and the Amounts of CO-Detectable Heme Remaining Following the Mechanism-Based Inactivation of CYP2B6 by Chlorpyrifos^a

incubation time (s)	% activity	% CO-P450
0	100%	100%
15	45%	44%
30	30%	23%
45	20%	13%

^aCYP2B6 (1 μ M) in the reconstituted system and in the presence of cytochrome *b*₅ (1 μ M) was inactivated at 37 °C for 45 s in the presence of 100 μ M chlorpyrifos. The activities remaining for the inactivated CYP2B6 were determined by analyses of the 7-EFC O-deethylase activities in the secondary reaction mixture, and the CO-detectable heme was measured by recording the visible spectrum of the ferrous CO-P450 complex after the addition of a few grains of dithionite as described under Materials and Methods. The results presented are the average of two experiments with duplicate measurements at each time point. The decreased activity was consistent with the amount of activity lost over time in inactivation kinetic experiments presented in Table 1 and Figure 1.

Table 4.

Summary of the 7-EFC O-Deethylase Activities and Native Heme Remaining Following the Mechanism-Based Inactivation of CYP2B6 by Chlorpyrifos without Cytochrome *b*₅ Added^a

	% activity lost	native heme (% lost)
CYP2B6 – NADPH	0	0
CYP2B6 + NADPH	58 ± 8.2%	63 ± 7.0%
CYP2B6 + NADPH + DDT	63%	73%

^aCYP2B6 (1 μ M), in the absence of cytochrome *b*₅, was inactivated at 37 °C for 5 min in the presence of 10 μ M chlorpyrifos. The activities remaining for the inactivated CYP2B6 were determined by analyses of the 7-EFC O-deethylase activities in the secondary reaction mixture, and the native heme was determined by HPLC, as described under Materials and Methods. The results for CYP2B6 without the addition of DDT are the average of three experiments presented with standard deviation. The results for CYP2B6 with the addition of DDT represents one experiment.