Thiophene Derivatives as New Mechanism-Based Inhibitors of Cytochromes P-450: Inactivation of Yeast-Expressed Human Liver Cytochrome P-450 2C9 by Tienilic Acid†

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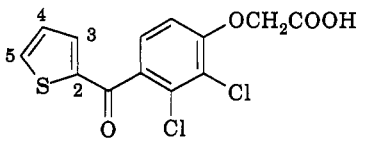
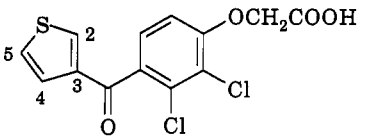
ABSTRACT: Oxidation of tienilic acid (TA) by microsomes of yeast expressing two closely related human liver cytochrome P-450s (P450), P450 2C9 and 2C10, led to catalysis-dependent loss of activity of these P450s. Under identical conditions, oxidation of a tienilic acid isomer (TAI) failed to give any P450 inactivation. The loss of P450 activity during TA oxidation was concomitant with product (5-hydroxytienilic acid, 5-OHTA) formation, showed pseudo-first-order and saturation kinetics, and was inhibited by an alternative substrate, tolbutamide. Covalent binding of TA metabolites to microsomal proteins occurred in parallel with enzyme inactivation and was partially inhibited by the presence of glutathione in the reaction medium. However, glutathione did not protect P450 enzyme from inactivation. Thus, TA exhibited all of the characteristics of a mechanism-based inactivator for P450 2C9 and 2C10 enzymes. The following kinetic parameters were determined in the case of P450 2C10: $t_{1/2,max}$ = 3.4 min, k_{inact} = 3.6 10^{-3} s^{-1}, K_I = 4.3 μM, k_{inact}/K_I = 813 L mol^{-1} s^{-1}, and partition ratio = 11.6. Moreover, a specific covalent binding of 0.9 mol of TA metabolite per mole of P450 2C10 was found to occur before the complete loss of enzyme activity (in incubations performed in the presence of glutathione). A plausible mechanism for P450 2C10 (2C9) inactivation during TA oxidation is proposed. It involves the intermediate formation of an electrophilic thiophene sulfoxide, which may react at position 5 of its thiophene ring either with H_2O to give 5-OHTA or with a nucleophilic group of an amino acid residue of the P450 active site, which results in its covalent binding to P450 protein. This alkylation and inactivation of P450 2C9 (2C10) by TA could be a starting point for the appearance of anti-P450 2C antibodies detected in patients treated with TA and suffering from immunoallergic hepatitis.

Many compounds, such as alkenes, alkynes, strained cycloalkylamines, 4-alkyldihydropyridines, benzodioxoles, and some tertiary amines, act as mechanism-based inactivators of cytochrome P-450s[1] [for a review, see for instance Ortiz de Montellano (1988) or Walsh (1982)]. Recently, it has been found that some thiophene derivatives are oxidized by liver P450s into the corresponding reactive thiophene sulfoxides (Mansuy et al., 1991). These electrophilic metabolites rapidly react with nucleophilic groups either from glutathione, with the eventual formation of mercapturic acid metabolites, or from liver proteins, which leads to their covalent binding to these proteins. For example, tienilic acid (TA) and its isomer (TAI) bearing the arylketo group on position 3 of the thiophene ring (Figure 1) have been found to be activated by rat liver (Dansette et al., 1990) and human liver (Dansette et al., 1991) P450s, with the formation of reactive metabolites that covalently bind to liver proteins. Tienilic acid (TA) is a uricosuric diuretic drug that has been found to cause immunoallergic hepatitis in about 1 out of every 10 000 patients treated (Homberg et al., 1984). TA-induced immunoallergic hepatitis has been associated with the appearance of circulating anti-organelle autoantibodies, called anti-LKM_2 antibodies, which are directed against a protein of human liver endoplasmic reticulum (Homberg et al., 1984). These anti-LKM_2 antibodies were later identified as specifically directed against a human liver P450 from the 2C subfamily that was also found to be responsible for the oxidative metabolism of TA in human liver microsomes (Beaune et al., 1987; Dansette et al., 1991). P450 2C-mediated TA metabolization by human liver microsomes leads to the formation of 5-hydroxytienilic acid (5-OHTA) as a major stable metabolite (Dansette et al., 1991). In the course of this hydroxylation, TA is also metabolically activated to electrophilic reactive intermediates which irre-



Tienilic Acid (TA)



Tienilic Acid Isomer (TAI)

FIGURE 1: Chemical structures of tienilic acid and its isomer.

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[1] Abbreviations: P450, cytochrome P-450; TA, tienilic acid; 5-OHTA, 5-hydroxytienilic acid; TAI, tienilic acid isomer (see Figure 1); anti-LKM_2, anti-liver kidney microsome type 2 antibodies; DETAPAC, diethylenetriaminepentaacetic acid; GSH, reduced glutathione.

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versibly bind to liver microsomal proteins (Dansette et al., 1991).

All of these data allowed us to propose a possible sequence of events describing the molecular mechanism for the development of TA-induced immunoallergic hepatitis (Beaune et al., 1987; Mansuy, 1991). This mechanism involves (i) the oxidation of the thiophene ring of TA into reactive metabolites by a P450 2C enzyme, (ii) the alkylation of this P450 by the reactive metabolite(s), (iii) the appearance of antibodies against this modified protein, and (iv) the destruction of hepatocytes bearing P450 2C–anti-P450 2C immune complexes by the immune system.

Very recently, it was shown that P450 2C9, one of the major P450s present in human liver, when expressed in yeast (Brian et al., 1989a,b; Srivastava et al., 1991), was able to reproduce the characteristic features of TA (and TAI) oxidation by human liver microsomes, i.e., 5-OHTA formation as a major pathway with metabolic activation of TA to electrophilic intermediates that irreversibly bind to microsomal proteins (López-Garcia et al., 1993). These results clearly showed that TA 5-hydroxylation and TA metabolic activation to electrophilic intermediates are both catalyzed by the same P450 enzyme. This study (López-Garcia et al., 1993) also strongly suggested that P450 2C9 is the major isozyme responsible for TA oxidation in human liver. Thus, yeast expressing human P450 2C9 (or the closely related P450 2C10) provides a unique experimental tool to investigate the mechanism and biological consequences of protein alkylation by reactive TA intermediates upon microsomal TA oxidation and its relationship to the appearance of anti-LKM_2 autoantibodies.

Although TA was shown to be activated by P450 2C9 (and P450 2C10) to alkylating metabolites, subsequent inactivation of this P450 form as a consequence of alkylation was not demonstrated. This article reports that during the oxidation of TA catalyzed by P450 2C9 (or 2C10), a loss of enzymatic activity, which exhibits the specific criteria for a mechanism-based P450 inactivation process, is occurring. These data indicate for the first time that certain thiophene derivatives could constitute a new class of suicide substrates for P450s. Kinetic characterization of the reaction shows that TA is in fact a rather efficient active-site-directed suicide substrate for P450 2C9/10.

EXPERIMENTAL PROCEDURES

Chemicals. Tienilic acid (TA) and its isomer (TAI) were provided by Anphar-Rolland (Chilly-Mazarin, France). [^{14}C]-TA and [^{14}C]TAI (label in the keto group) were prepared by CEA (Saclay, France) and Amersham (Bucks, U.K.), respectively. Their radiochemical purity was checked by HPLC and found to be higher than 98%.

Yeast Transformation, Cell Culture, and Preparation of the Yeast Microsomal Fraction. The isolation and sequencing of the human cDNA MP-4 (2C9) and MP-8 (2C10) clones have been previously reported (Umbenhauer et al., 1987; Ged et al., 1988; Brian et al., 1989a). These DNA sequences were inserted into the pAAH5 vector (based on an ADH1 promoter and terminator) that contains the *leu2* gene. *Saccharomyces cerevisiae* strain D12 [a, leu$^-$, (cir$^+$)] was used for expression. Details of the protocols for construction of vectors, yeast transformation, and screening for selection are presented elsewhere (Brian et al., 1989a,b; Srivastava et al., 1991).

These transformed yeasts, referred to in the text as pAAH5/P450 2C9 and pAAH5/P450 2C10, were cultured semi-anaerobically at 28 °C in synthetic minimal medium [0.67% (w/v) Difco yeast nitrogen base without amino acids and 2% (w/v) glucose] supplemented with amino acids (except leucine). Growth was monitored carefully by light scattering at 600 nm, and the cultures were allowed to reach an A_{600} of 1.7–1.8 (about 5×10^7 cells/mL), at which time culture was stopped by quick refrigeration. Cells were harvested by centrifugation (5000*g*, 10 min), pooled, and washed twice with $^1/_{10}$ vol of distilled water. Yeast cell pellets were stored at −80 °C until they were processed for microsome preparation. Routinely, yeasts from 5 L of culture were pooled and processed simultaneously.

Yeast microsomes were prepared as previously described (Brian et al., 1989b; Guengerich et al., 1991) using yeast lytic enzyme (Sigma) for cell wall digestion followed by sonication. The final microsomal pellet was homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (v/v), aliquoted, frozen under liquid N_2, and stored at −80 °C until use. P450 recovery in the microsomal fraction was similar for both yeast constructs (40% of the initial P450 hemoprotein, as determined in the intact whole yeast).

General Assays. Quantitation of 5-OHTA in aliquots (routinely 140 μL) from the different experiments described below was based on a kinetic spectrophotometric method (Neau et al., 1990) adapted to an end-point assay, as previously described (López-Garcia et al., 1993). To determine the covalent binding of TA- and TAI-reactive metabolites to microsomal proteins, aliquots from the different experiments (routinely 50 μL) were immediately processed as described previously (Dansette et al., 1990). Microsomal P450 content was determined according to Omura and Sato (1964). The protein content in microsomal suspensions was determined by the Lowry procedure (Lowry et al., 1951) using bovine serum albumin as a standard.

Inactivation Experiments. (A) General Incubation Conditions. Incubations for metabolic activity with yeast microsomes were always carried out in duplicate at 28 °C, using glass tubes in a shaking bath. The incubation mixtures contained the yeast microsomal suspension (pAAH5/P450 2C9 or pAAH5/P450 2C10 yeast microsomes, providing 0.3 μM P450), the substrate (as indicated in the experiments), and a NADPH-generating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, and 2 units of glucose 6-phosphate dehydrogenase/mL) diluted in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM DETAPAC and 8% glycerol (final concentrations). Activity assays were routinely initiated (t_0 = 0 min) by incorporation of the NADPH-generating system into the incubation mixture after 3 min of separate preincubation at 28 °C for temperature equilibration. These conditions apply, unless noted otherwise, to all of the experiments described below.

(B) Time Course Analysis of TA and TAI Microsomal Oxidation. In these experiments, incubations were carried out in the general conditions described above, but in the presence of a saturating substrate concentration (100 μM [^{14}C]TA, 43 mCi/mmol, or 100 μM [^{14}C]TAI, 25 mCi/mmol; label in the keto group). GSH, when added, was 5 mM (final concentration). At t_0 (0 min) and regularly thereafter, aliquots were taken and quickly processed for the determination of either covalent binding of TA (or TAI) metabolites to microsomal protein (50 μL) or 5-OHTA formation (140 μL), as reported previously (López-Garcia et al., 1993).

(C) Incubations for Inactivation Kinetics. The experimental design to determine the rate of enzyme inactivation was based on the general procedures previously described for other P450 suicide substrates in liver microsomes (Halpert & Stevens,

1991; Underwood et al., 1992). Basically, yeast microsomes (equivalent to 0.3 μM P450) were incubated in the conditions outlined above with 5 mM GSH and in the presence of variable concentrations of TA (ranging from 3 to 100 μM). At t_0 and regularly thereafter (from 0 to 30 min), parallel aliquots were removed from the incubation and were immediately processed to determine 5-OHTA formation (140 μL) and residual enzymatic activity (300 μL), as will be described later. The same procedure was also used in experiments aimed at correlating the time and TA concentration dependence of 5-OHTA formation with enzyme inactivation. Experiments designed to determine the time dependence of P450 2C9/10 enzyme activity upon TAI oxidation were similar overall, except that only TAI-saturating concentrations (100 μM [^{14}C]-TAI, 25 mCi/mmol) were tested. Covalent binding of TAI metabolites was determined in this case as a measure of TAI oxidation in parallel with the determination of the enzyme activity remaining (described below). In all instances, control incubations were run in parallel with the experimental variables to correct for the possible contribution of microsomal lability to the time-dependent loss of activity. For that purpose, yeast microsomes were incubated in the general conditions described but under noncatalysis conditions, i.e., either in the presence of substrate (100 μM TA) but not the NADPH-generating system or in the presence of NADPH but not the substrate. Aliquots were taken regularly (from t_0) for the immediate determination of the remaining monooxygenase activity (as will be described later).

(D) Measurement of the Remaining Enzymatic Activity. Routine experimental procedures to determine the enzymatic activity remaining upon exposure to a suicide substrate (Halpert & Stevens, 1991; Underwood et al., 1992) incorporate the use of an alternative substrate to assay the enzyme activity in a second incubation period. This requires the prior elimination of the inactivator substrate already present (in excess and/or at different concentrations) in the aliquots, which otherwise could interfere in the accurate determination of enzyme residual activity (i.e., competitive inhibition and enzyme inactivation are superimposed). This is routinely achieved (Halpert & Stevens, 1991; Underwood et al., 1992) by "washing off" the excess substrate (quick dilution of the aliquot in buffer, sedimentation of the microsomal pellet at 100000*g*, and resuspension in the standard assay medium) or by 10–100-fold dilution of the sample in the standard assay medium, which minimizes the interference of the inactivator with the second substrate.

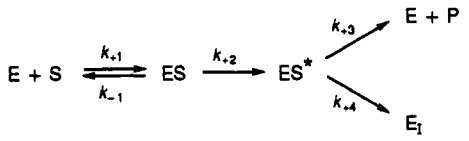
The particular characteristics of the yeast microsomal suspension (relative instability under the hard conditions necessary for the "washing off" procedure and a low level of P450 expression per milligram of microsomal protein) prevented manipulation of the aliquots removed from the incubations for inactivation by either the washing off or the dilution procedure. Because of the high affinity of P450 2C9/10 enzymes for TA as substrate (K_m = 6 μM; López-Garcia et al., 1993) compared to tolbutamide (the typical substrate for these enzymes, whose apparent K_i for TA 5-hydroxylation is 1 mM; López-Garcia et al., 1993), the excess TA in the medium precludes the use of tolbutamide hydroxylation as the control reaction to accurately determine the residual enzyme activity. Thus, similar to studies in which the inactivator is known to be a substrate for the enzyme and product formation can be monitored (Jones & Fitzpatrick, 1990), TA 5-hydroxylation was determined as a control reaction for residual enzyme activity in a second incubation in which conditions for maximal initial rates were provided. Briefly, 100 μM TA was quickly added to 300-μL aliquots from the inactivation experiment in order to achieve a saturating substrate concentration. GSH was also added in case it was not present in the incubated mixtures (final concentration, 5 mM). An aliquot (140 μL) was taken and immediately quenched by treatment with 70 μL of a cold CH_3CN/CH_3COOH (10:1) mixture. The remaining medium was incubated for 5 min, and a second aliquot (t_5, 140 μL) was removed and treated as the first one. The rate of 5-OHTA formation was determinated as previously reported (López-Garcia et al., 1993): 5-OHTA formation was determined by recording the difference spectrum ($A_{390}-A_{490}$) of the t_5 sample against a base line performed with the t_0 sample ($\epsilon_{390-490}$ = 28 000 M^{-1} cm^{-1}) using 100-μL black-masking quartz microcuvettes (Hellma, Mulheim, Germany). This procedure applies for all of the determinations noted as "activity remaining" in the referenced experiments. In all instances, the initial TA 5-hydroxylation rate (determined at t_0 of incubation and in the presence of 100 μM TA and a NADPH-generating system in all studies) was taken as a measure of the maximal enzyme activity (i.e., 100% activity). Thus, the percent activity remaining after a given incubation time with a given TA (or TAI) concentration is a fraction of the corresponding initial (100%) activity.

(E) Protective Effect of Tolbutamide. To determine the time course of enzyme inactivation in the presence of tolbutamide, incubations were carried out as described in section C, but a lower TA concentration (5 μM) was chosen for inactivation. Tolbutamide (1 mM) (on the basis of its apparent K_i for P450 2C10-mediated TA 5-hydroxylation) was added (or not) at the t_0 of exposure to 5 μM TA. At the indicated times, 5-OHTA formation and the residual enzyme activity were determined in parallel aliquots (140 and 300 μL, respectively), as described above. No significant differences were found in the maximal initial hydroxylation rate (100% at t_0 incubation), whether or not tolbutamide was present in the assay for residual activity (1.74 ± 0.02 nmol of 5-OHTA/nmol of P450/min vs 1.68 ± 0.05 nmol of 5-OHTA/nmol of P450/min with and without tolbutamide, respectively). Controls (under noncatalysis conditions) were also run in parallel with the experimental incubations.

(F) Effect of GSH on the Rate of Inactivation. The time dependence determination of P450 inactivation upon oxidation of 10 μM TA was performed according to the general procedure described, with or without 5 mM GSH present in the incubation mixture. The percent enzyme activity remaining was monitored in the standard way by aliquots taken regularly (from 0 to 30 min) from the incubation mixture. Control incubations containing the NADPH-generating system with and without 5 mM GSH but with no TA added were also run in parallel.

(G) Stoichiometry of the Inactivation. Yeast microsomes were incubated under standard conditions in the presence of 5 mM GSH and 10 μM [^{14}C]TA (43 mCi/mmol). Inactivation was allowed to proceed until the residual activity was minimal (about 30 min). During the course of this period, aliquots were taken regularly in which covalent binding of TA metabolites to protein (50 μL) and the remaining enzyme activity (300 μL) were determined in parallel by the described procedures.

(H) Kinetic Analyses. Kinetic parameters of the inactivation process were calculated according to the models of Walsh (1982) and Walley (1980), later reviewed by Tipton (1989); this process can be represented in the simplest way by the following equation:



where ES is the initial enzyme–substrate complex converted to an activated species, ES*. S* (the electrophilic TA intermediate) can either react within the complex to produce E_I (the irreversibly inhibited enzyme) or break down to yield free enzyme (E) and products (P). k_{-1}/k_{+1} represents the K_I (dissociation constant), and k_{+3}/k_{+4} is the partition ratio between the number of productive turnovers and those leading to enzyme inactivation. The pseudo-first-order rate constant for the inactivation process (k_{inact}) is $k_{+2}k_{+4}/k_{+2} + k_{+3} + k_{+4}$. Data presented here were collected over the course of several months with five different microsomal preparations. Curve fitting was performed by a single regression fit procedure of Cricket Graph (Cricket Software, Malvern, PA) on a Macintosh SE personal computer.

RESULTS

Preliminary Evidence for the Time-Dependent Loss of P450 2C10 (2C9) Catalytic Activity during Hydroxylation of TA. A careful analysis of the time course evolution of TA 5-hydroxylation by microsomes from yeast expressing P450 2C10 showed a short linear period (ca. 5 min) followed by a fast decrease of the reaction rate (Figure 2A). In fact, after a 30-min period, no further significant hydroxylation of TA could be detected, even if more substrate was added to the incubation mixture (data not shown). A very similar decrease as a function of time was observed for the activation of TA into reactive metabolites able to bind covalently to microsomal proteins (Figure 2B). On the contrary, under identical conditions, oxidation of TAI into reactive metabolites which covalently bind to proteins remained linear as a function of time for at least 20 min (Figure 2C). The covalent binding of TA metabolites to proteins, which is concomitant with TA hydroxylation, was considerably reduced when microsomal incubations were performed in the presence of nucleophiles like glutathione (Figure 2B). However, a residual level of covalent binding that remained insensitive to the trapping agent was observed even in the presence of 5 mM GSH, the maximal effective concentration (Figure 2B). Interestingly, the time-dependent decrease of P450 2C10-catalyzed 5-OHTA formation was the same in the presence or absence of 5 mM GSH (Figure 2A). It is noteworthy that almost identical results were obtained by using pAAH5/P450 2C9 microsomes instead of pAAH5/P450 2C10 under identical conditions (not shown). These preliminary results first suggested that the residual covalent binding of TA metabolites, which was observed in the presence of excess GSH, could be responsible for the inactivation of the P450 enzyme during TA oxidation.

To further analyze this hypothesis, yeast microsomes were incubated with either TA or TAI in the presence and absence of NADPH (i.e., under catalysis and noncatalysis conditions), and the remaining enzyme activity was measured as a function of time. After incubation with TA or TAI in the absence of an NADPH-generating system (Figure 3A,B), the activity of pAAH5/P450 2C10 microsomes remained constant (100%) for at least 30 min. It underwent a 10% decrease only after a 45–60-min period (not shown), which indicates that yeast microsomal suspensions are rather stable under the incubation conditions used in our experiments. Similar results were obtained with incubations performed with the NADPH-generating system but in the absence of substrate (TA or TAI) or when pAAH5/P450 2C9 microsomes were used instead of pAAH5/P450 2C10 (data not shown). When microsomes were incubated in the presence of NADPH and TAI (100 μM), and in the absence of GSH even in order to maximize any inactivating effect, no significant loss of activity could be detected between 0 and 15 min of incubation (Figure 3B), with the 30% decrease in activity observed after 30 min being explained by the high level of protein alkylation by reactive TAI metabolites already achieved at that time (Figure 2C). On the contrary, when microsomes were incubated in the presence of NADPH and TA (100 μM) and 5 mM GSH (to minimize covalent binding to proteins), a dramatic loss of activity was observed (Figure 3A). Identical results were obtained with pAAH5/P450 2C9 yeast microsomes (Figure

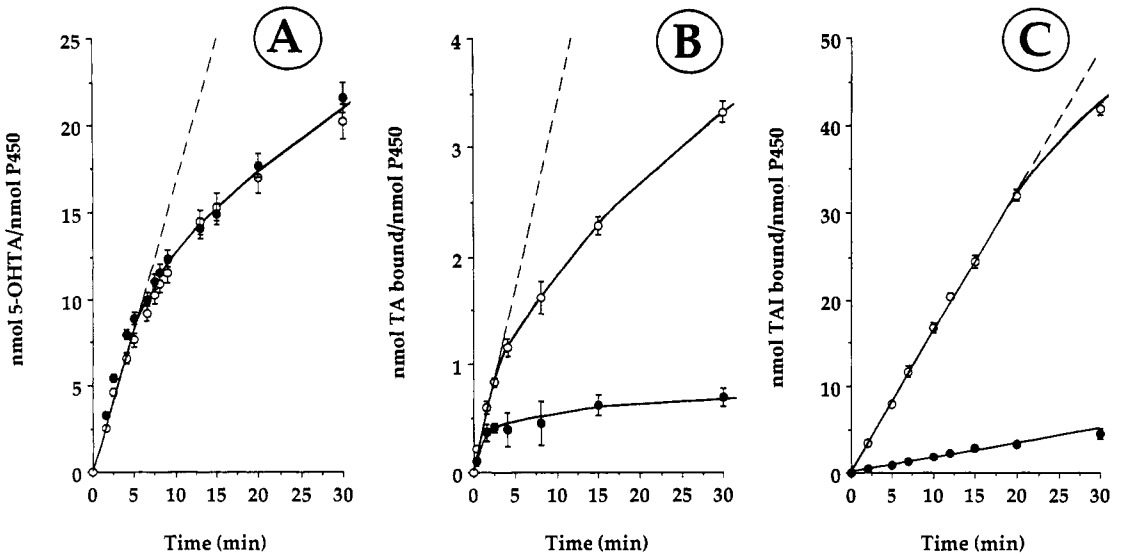


FIGURE 2: Microsomal oxidation of TA and TAI by yeast-expressed human P450 2C10 in the presence (●) and absence (○) of 5 mM GSH, as a function of time. The time course of TA 5-hydroxylation (A) and covalent binding of TA-reactive metabolites to microsomal proteins (B), in parallel aliquots from the same incubation, as well as the covalent binding of TAI metabolites to microsomal proteins (C) was determined as described in Experimental Procedures. Values are the mean ± SD from three independent experiments. From linear regression analysis of the corresponding initial linear period (– –), mean catalytic rates were estimated to be 1.66 nmol of 5-OHTA/nmol of P450/min, 0.33 nmol of TA bound/nmol of P450/min, and 1.61 nmol of TAI bound/nmol of P450/min, respectively.

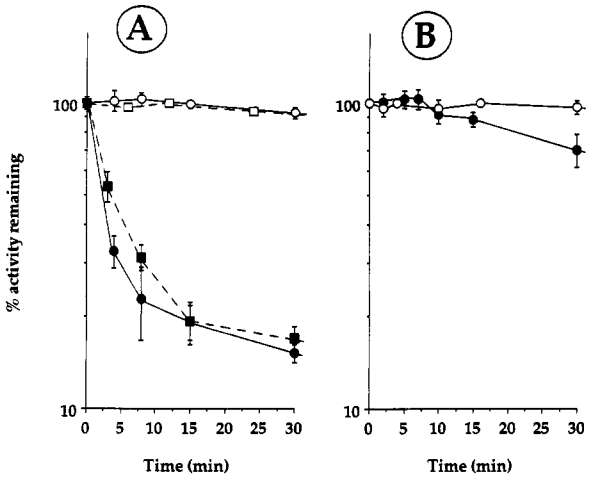


FIGURE 3: Time dependence of P450 2C9/10 inactivation after NADPH-dependent oxidation of TA (A) and TAI (B) by yeast-expressed human P450 2C9/10. The residual TA 5-hydroxylation activity was measured as described in the Experimental Procedures, following different incubation times under the indicated conditions: (A) pAAH5/P450 2C9 (□, ■) or pAAH5/P450 2C10 (○, ●) yeast microsomes incubated with 100 μM [^{14}C]TA plus 5 mM GSH, in the presence (■, ●) and absence (□, ○) of an NADPH-generating system (100% values are 1.65 ± 0.05 and 1.42 ± 0.02 nmol of 5-OHTA/nmol of P450/min for P450 2C10 and P450 2C9, respectively); (B) pAAH5/P450 2C10 yeast microsomes incubated with 100 μM [^{14}C]TAI, in the presence (●) and absence (○) of an NADPH-generating system (100% value was 1.44 ± 0.05 nmol of 5-OHTA/nmol of P450/min. Values are the mean ± SD from three independent experiments.

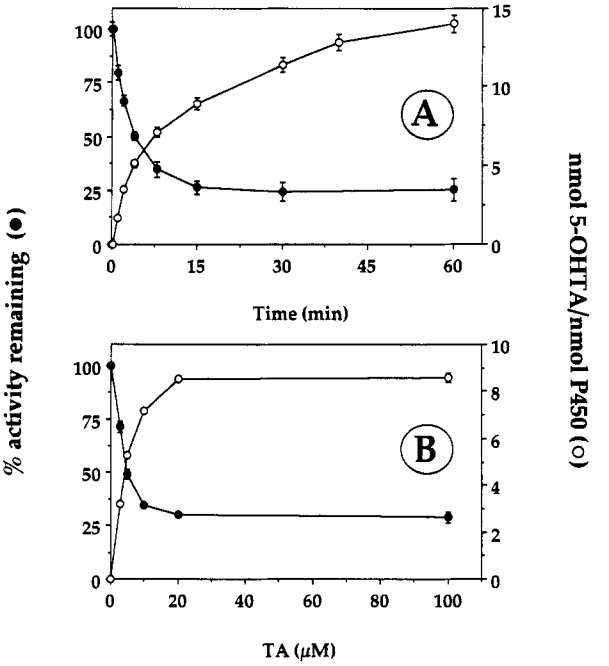


FIGURE 4: Relationship between product (5-OHTA) formation (○) and P450 2C10 residual activity (●) upon oxidation of TA by P450 2C10, as a function of time (A) and TA concentration (B). 5-OHTA formation and the remaining P450 2C10 activity were determined in parallel, as detailed in the Experimental Procedures. (A) pAAH5/P450 2C10 yeast microsomes were incubated for the indicated times in the presence of NADPH, 5 mM GSH, and 10 μM TA. (B) pAAH5/P450 2C10 yeast microsomes were incubated for 8 min with NADPH, 5 mM GSH, and the indicated TA concentrations. Values are means ± SD from three independent experiments.

3A). These results confirmed the existence of catalysis-dependent loss of P450 2C10 (2C9) monooxygenase activity upon TA (but not TAI) oxidation. The general criteria (Walsh, 1982; Walley, 1980; Tipton, 1989) to prove that irreversible loss of activity upon TA oxidation is a mechanism-based process were then studied.

Kinetics of the Inactivation Process. Figure 4A shows that the loss of P450 2C10 activity paralleled 5-OHTA formation. For a given time of incubation, the loss of activity was found to be dependent on TA concentration, with saturation kinetics similar to that observed for 5-OHTA formation (Figure 4B). Loss of activity as a function of time for microsomes incubated in the presence of NADPH and various concentrations of TA (Figure 5A) was exponential with time and showed the classic biphasic kinetics already described (Halpert & Stevens, 1991; Manno et al., 1991) for other P450 suicide substrates. Since most of the inactivation at a given TA concentration had occurred within 5 min, only the early time points were included in the kinetic analyses of the inactivation. The time required for half-maximal inactivation ($t_{1/2}$) at each substrate concentration, as well as the apparent first-order rate constant, k_{inact}, was calculated from the plot of the logarithmic transformation of the remaining activity as a function of time, as depicted in Figure 5B. Plots of the $t_{1/2}$ vs the reciprocal of TA concentration (linear, Figure 5B, inset) and plots of the observed k_{inact} vs TA concentration (hyperbolic, not shown) led to an estimation of the kinetic constants that define the inactivation process. From extrapolation to infinite TA concentration, the time required to inactivate one-half of the enzyme at the maximal rate ($t_{1/2max}$) and the maximal k_{inact} were, respectively, 3.4 ± 0.8 min and (3.6 ± 0.8) × 10^{-3} s^{-1} (n = 3). The dissociation constant (K_I) was found to be 4.3 ± 0.6 μM, a value not significantly different from the K_m value for TA 5-hydroxylation determined with several microsomal preparations (5.9 ± 2.3 μM). Determination of the second-order rate constant (k_{inact}/K_I), a proposed index of the "in vitro effectiveness of the substrate as inactivator" (Bednarsky & Nelson, 1989), gave a mean value of 813 ± 80 L mol^{-1} s^{-1}.

An interesting parameter in the characterization of a mechanism-based inhibitor is the so-called partition ratio, r (k_{+3}/k_{+4} in the kinetic scheme illustrated in the Experimental Procedures section), which represents the number of productive turnovers (leading to 5-OHTA in this case) divided by the number of inactivating events. Under conditions in which complete inactivation is achieved, r corresponds to the P_∞/E_0 ratio, where E_0 is the initial enzyme concentration and P_∞ is the final concentration of product formed (Walley, 1980). From extrapolation to infinite time in time course experiments in which the formation of 5-OHTA from different TA concentrations was monitored (as in Figure 4A), a partition ratio of 11.8 ± 2.7 was calculated (not shown). A more accurate value for r was derived from Figure 6, which correlates the percent activity remaining after TA oxidation with the amount of 5-OHTA formed during the same time. A linear relationship was observed, regardless of the TA concentration or the incubation time. Extrapolation to 0% remaining activity gave a mean r value of 11.6 ± 0.2 (n = 3), in good agreement with the value estimated above. The same kind of experiments and calculations made on pAAH5/P450 2C9 microsomes gave an almost identical r value (Figure 6).

Tolbutamide Protection from P450 Inactivation by TA. In a mechanism-based process, the presence of a competitive inhibitor of the inactivator substrate must protect the enzyme from inactivation, since binding to the active site always precedes inactivation (Walsh, 1982). As expected, the presence of tolbutamide, a known substrate for P450 2C9/10 enzymes which has been characterized as a competitive inhibitor for TA 5-hydroxylation (López-Garcia et al., 1993),

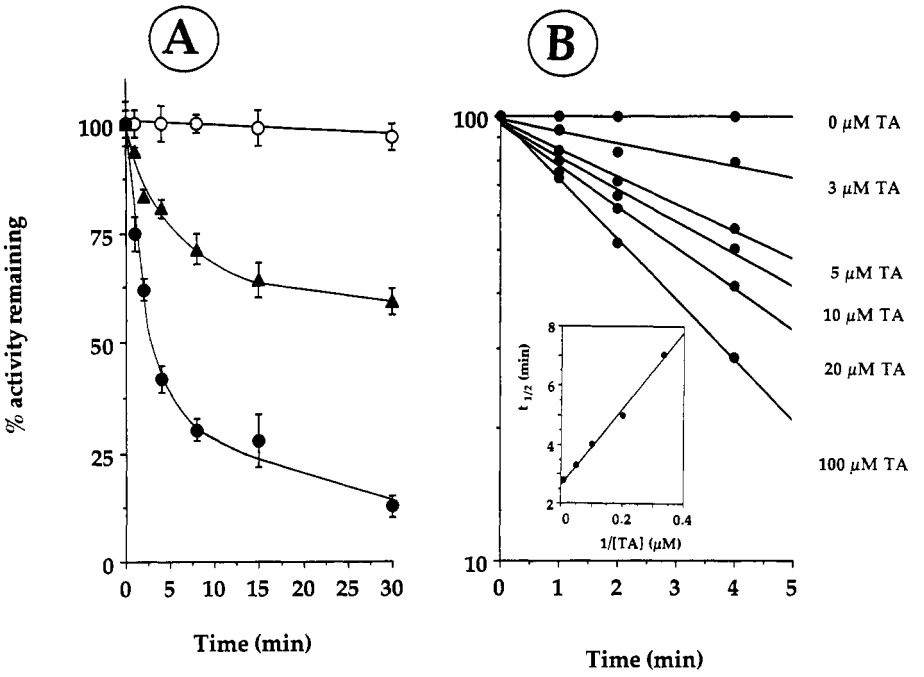


FIGURE 5: Kinetics of P450 2C10 inactivation by different concentrations of TA. Details for incubation and determination of the remaining activity have already been given in the Experimental Procedures section. (A) pAAH5/P450 2C10 yeast microsomes were incubated for the indicated periods in a mixture which contained the NADPH-generating system, 5 mM GSH, and 0 (○), 3 (▲), or 20 μM (●) TA. Data correspond to the mean ± SD of three independent experiments. (B) Linear regression analysis of the natural logarithm of the remaining P450 2C10 activity as a function of time allowed the calculation of the time required for half-inactivation of the enzyme upon incubation in the presence of varying TA concentrations (as indicated). The points shown are for individual data from a representative experiment out of three. Inset: Plot of the estimated $t_{1/2}$ vs the reciprocal of the TA concentration for the experiment depicted (r^2 = 0.991 for this correlation).

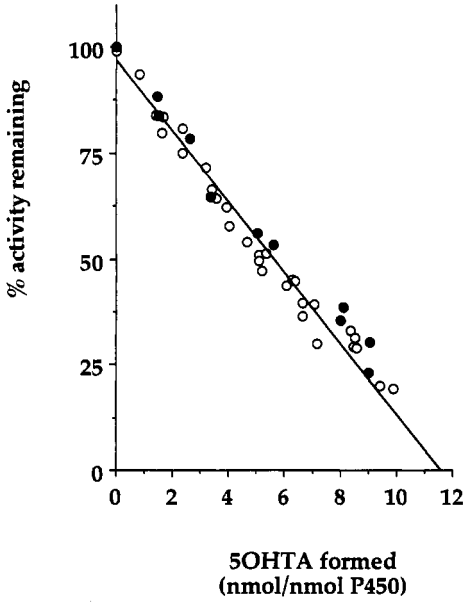


FIGURE 6: Correlation between mechanism-based P450 2C9/10 inactivation and efficient catalysis (5-OHTA formation) upon TA oxidation. Microsomes from yeasts expressing P450 2C9 (●) and P450 2C10 (○) were incubated under catalysis conditions in the presence of varying concentrations of TA (from 3 to 100 μM) and for different time periods (from 0 to 15 min). The amount of 5-OHTA formed, as well as the remaining monooxygenase activity, was determined in parallel aliquots. Points are individual values from three different experiments for P450 2C10 and two separate experiments for P450 2C9. As shown, regardless of the time or the TA concentration used, the percent remaining activity correlated linearly with the amount of 5-OHTA formed in the corresponding period [$y = 97.8 - 8.37x$ ($r^2 = 0.98$) for P450 2C10 as depicted, and $y = 97.0 - 7.85x$ ($r^2 = 0.98$) for P450 2C9, not shown].

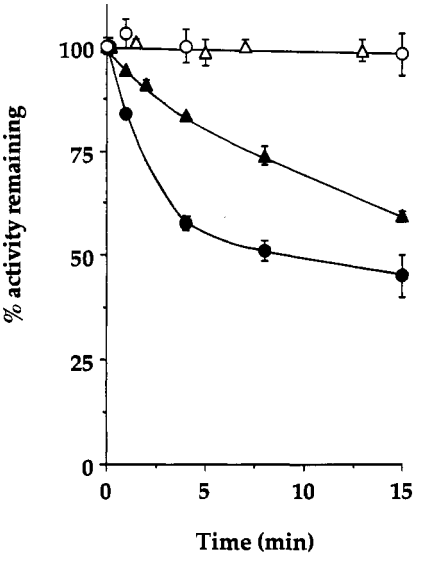


FIGURE 7: Protection by tolbutamide of P450 2C10 inactivation upon TA oxidation. pAAH5/P450 2C10 yeast microsomes were incubated in the presence of NADPH, with either 5 μM TA (●) or 5 μM TA plus 1 mM tolbutamide (▲) for the indicated periods. After this first incubation, the percent remaining enzyme activity was determined as indicated in the Experimental Procedures. Controls in noncatalysis conditions (without an NADPH-generating system added) were also run in parallel (○, △). Data are means ± SD.

inhibited both 5-OHTA formation and P450 inactivation (Figure 7). This was reflected by a significant increase in the $t_{1/2}$ of the enzyme when tolbutamide was present (from 5.0 to 20.3 min, i.e., a 4-fold increase, in the experiment showed). Thus, tolbutamide can delay, but not completely prevent, the TA-dependent inactivation of P450.

Effect of GSH on the Inactivation Rate. One key property of an efficient mechanism-based inhibitor is the ability of the reactive species formed by enzymatic activation to react more rapidly within the enzyme active site than to diffuse out into the solution. If not, the reactive species could react with other

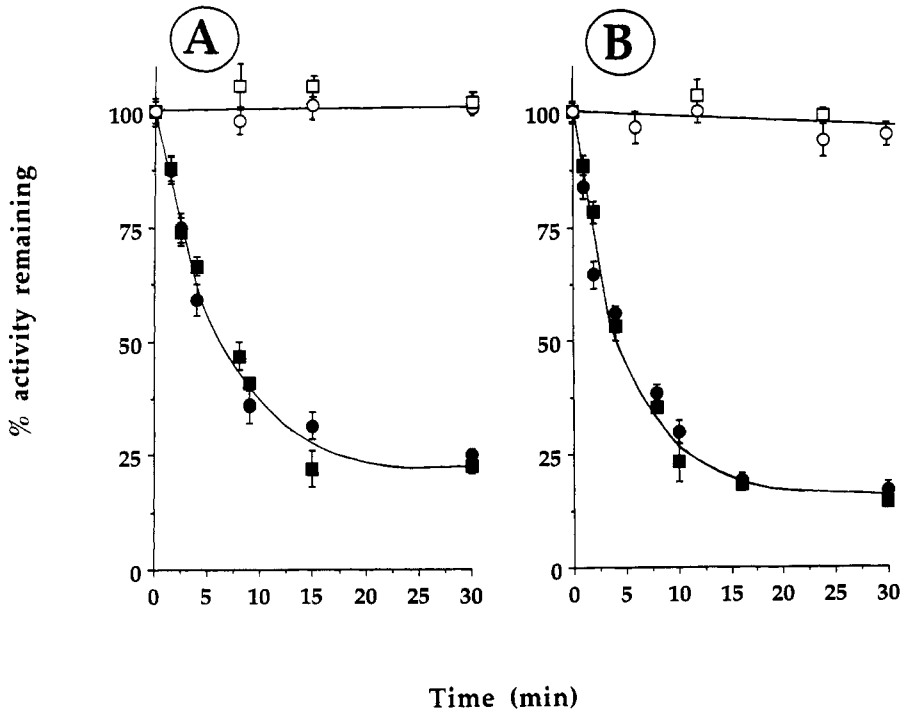


FIGURE 8: Effect of GSH on the rate of P450 2C10 (A) and P450 2C9 (B) inactivation upon TA oxidation. Loss of P450 2C10 (A) and P450 2C9 (B) TA 5-hydroxylase activity after NADPH-dependent oxidation of 10 μM TA was evaluated as a function of time in the presence (●) and absence (■) of 5 mM GSH. Control incubations containing the NADPH-generating system with (○) or without (□) GSH but with no TA added were also run in parallel. Data are means ± SD from three experiments. The 100% values correspond to 1.52 ± 0.10 nmol of 5-OHTA/nmol of P450/min and 1.35 ± 0.05 nmol of 5-OHTA/nmol of P450/min for P450 2C10 and P450 2C9, respectively.

nucleophiles (e.g., proteins) in the medium, and enzyme inactivation could be due to such nonspecific alkylations outside the enzyme active site. Previous observations that GSH efficiently decreased the covalent binding of TA electrophilic intermediates to microsomal protein (Figure 2B) indicated that, in fact, part of these TA-reactive metabolites diffused out from the P450 2C10 (2C9) active site. However, as shown in Figure 8A, a 5 mM GSH concentration, which led to a maximum decrease in the covalent binding of TA metabolites to microsomal proteins, had no significant effect on the rate of P450 2C10 inactivation in the presence of TA and NADPH (identical $t_{1/2}$'s for inactivation with and without GSH of 5.2 ± 0.5 and 6.5 ± 0.9 min, respectively). Here too, equivalent results were obtained with pAAH5/P450 2C9 yeast microsomes (Figure 8B). The lack of a GSH effect on the inactivation kinetics suggested that the covalent binding which remained, even in the presence of excess GSH, occurred within the enzyme protein and was mainly responsible for P450 2C9/10 inactivation.

Relationship between P450 Alkylation and Inactivation. Time course experiments were carried out to correlate the rate of enzyme activity loss with the amount of [^{14}C]TA metabolite(s) covalently bound to microsomal protein. The experimental findings previously shown indicated that, in the presence of excess GSH, this covalent binding should mainly occur within the P450 2C9/10 active site. In agreement with this assertion, recent preliminary immunoblot data using a specific antibody against TA (covalently bound to albumin through its thiophene ring) showed that P450 2C9/10 was the main microsomal protein alkylated by TA metabolite(s) upon incubation of microsomes from yeast expressing P450 2C9 or 2C10 with TA and NADPH, in the presence of excess GSH (S. Lecoeur, E. Bonierbale, D. Challine, J. C. Gauthier, P. Valadon, P. M. Dansette, R. Catinot, F. Ballet, D. Mansuy, and P. Beaune, submitted for publication). As shown in Figure 9, P450 2C10 inactivation and covalent binding of TA

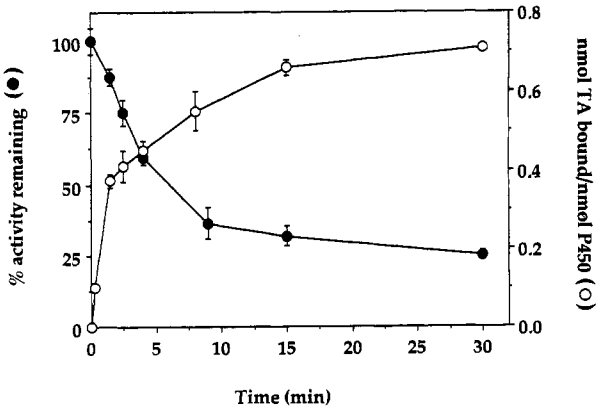


FIGURE 9: Relationship between P450 2C10 mechanism-based inactivation (●) and covalent binding of TA metabolites to protein (○) as a function of time. pAAH5/P450 2C10 yeast microsomes were incubated for different times in the presence of the NADPH-generating system, 5 mM GSH, and 10 μM [^{14}C]TA, as detailed in the Experimental Procedures. The covalent binding of TA metabolites to microsomal proteins and the remaining P450 2C10 monooxygenase activity were determined in parallel aliquots. Values are means ± SD of triplicate incubations.

metabolite(s) occurred simultaneously as a function of time. Approximately 0.7 nmol of TA metabolite(s) is covalently bound per nanomole of P450 when 80% enzyme inactivation has occurred. A plot of the percent remaining activity vs the amount of covalent binding (per nanomole of P450) led to a more accurate determination of the number of TA-activated molecules required to be covalently bound to P450 2C10 in order to cause a total loss of enzyme activity (Figure 10). Extrapolation for this curve showed that, in the presence of 5 mM GSH, total inactivation of P450 (0% remaining activity) was obtained when 0.9–1 nmol of the TA metabolite(s) was covalently bound per nanomole of P450. By contrast, in the absence of GSH about 3 nmol of TA-activated metabolite

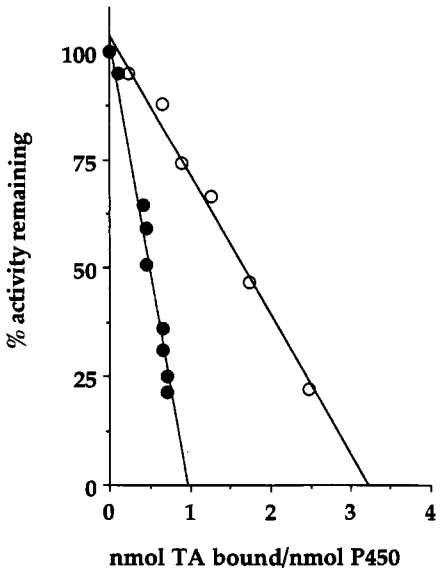


FIGURE 10: Correlation between P450 2C10 inactivation and covalent binding of TA metabolites to protein. pAAH5/P450 2C10 yeast microsomes were incubated under catalysis conditions for different periods and in the presence of variable concentrations of TA (from 3 to 20 μM), with (●) or without (○) 5 mM GSH added. The amount of TA metabolites covalently bound to microsomal proteins, as well as the P450 activity remaining, was determined in parallel aliquots. Points are individual data from two independent experiments. Regardless of the time or TA, a linear relationship could be established [$y = 103.8 - 32.3x$ ($r^2 = 0.98$) without GSH and $y = 102.7 - 107.0x$ ($r^2 = 0.98$) with GSH added].

(per nanomole of P450) was covalently bound to microsomal proteins when the enzyme became fully inactivated.

DISCUSSION

The aforementioned results clearly show that the catalysis-dependent loss of P450 activity observed during oxidation of TA by yeast-expressed human liver P450 2C9 (2C10) is coincident with TA 5-hydroxylation in a process that clearly meets the chemical and kinetic criteria generally accepted for selective, mechanism-based enzyme inactivation (Walsh, 1982; Walley, 1980; Tipton, 1989). Covalent binding of TA metabolite(s) to proteins occurs in parallel with enzyme inactivation. The presence of GSH in the medium markedly decreases the level of covalent binding of TA to microsomal proteins, although a residual binding is always observed and GSH has no effect on enzyme inactivation. The failure of GSH to decrease the inactivation rate strongly suggests that P450 inactivation (although involving electrophilic species that can diffuse to some extent to the surrounding medium) is due to a selective modification at the P450 active site after alkylation by TA-reactive metabolites and does not result from a nonspecific protein alkylation process.

Mechanism-based inactivation of an enzyme is linked to the covalent binding of a reactive intermediate to the enzyme active site in a 1:1 stoichiometry (Walsh, 1982; Fersht, 1977). Good evidence for mechanism-based P450 2C9/10 enzyme inactivation by selective protein alkylation at its active site is the 1:1 stoichiometry of covalent binding of TA metabolite(s) to P450 obtained for the complete loss of enzyme activity (Figure 10). From these results, it appears that TA exhibits all of the characteristics of a mechanism-based inhibitor of P450 2C9 and P450 2C10. The kinetic parameters of the process—i.e., the $t_{1/2max}$ (3.4 min), k_{inact} (3.6×10^{-3} s^{-1}), K_I (4.3 μM), k_{inact}/K_I (813 L mol^{-1} s^{-1}), and the partition ratio (about 12)—indicate that TA is a rather efficient suicide substrate when compared to other known inactivators of the same class for P450s or other enzymes (Walsh, 1982; Ortiz de Montellano, 1988).

It is also noteworthy that TAI, the isomer of TA bearing the arylketo group at position 3 of the thiophene ring instead of position 2 (Figure 1), does not act as a mechanism-based inhibitor of P450 2C9/10 enzymes. TAI is a competitive inhibitor of TA 5-hydroxylation (Dansette et al., 1990), which is metabolically activated to electrophilic intermediates by the same P450 2C that oxidizes TA (Dansette et al., 1991; López-Garcia et al., 1993). Metabolic activation and covalent binding of TAI metabolites to protein are the major features (80%) of TAI biotransformation in rat and human liver microsomes (Dansette et al., 1990, 1991). Interestingly, no loss of P450 2C10 (2C9) enzyme activity was found when transformed yeast microsomes catalyzed TAI oxidation (Figure 3B), despite the extensive covalent binding of TAI metabolite(s) to microsomal protein (42 nmol of TAI metabolite(s) per nanomole of P450 after 30 min of incubation under the conditions of Figure 2C). The different action of TA and TAI toward P450 2C9 (2C10) illustrates the fact that the mere formation of a reactive species at the active site of an enzyme does not imply that a chemical reaction with a nearby residue will occur.

The primary intermediate formed during P450-dependent oxidation of TAI has been recently identified as the thiophene sulfoxide (Mansuy et al., 1991). Even more recently, thiophene sulfoxide itself has been shown to be the primary reactive metabolite of thiophene in rats *in vivo* (Dansette et al., 1992). Thiophene sulfoxides are very electrophilic species which rapidly react with nucleophiles like thiols (Mansuy et al., 1991). This should explain the covalent binding of TAI metabolite(s) to microsomal proteins. It is likely that the P450-dependent oxidation of TA also leads to a thiophene sulfoxide as a primary intermediate, even though the detailed mechanism of the isomerization of TA sulfoxide into 5-OHTA remains to be elucidated.

A possible mechanism for the suicide inactivation of P450 2C9 (or 2C10) during TA oxidation is shown in Figure 11. The first step for both TA 5-hydroxylation and P450 inactivation could be the S-oxidation of the thiophene ring of TA. The thiophene sulfoxide of TA should be particularly electrophilic because of the presence of a keto substituent at position 2. A Michael-type addition of H_2O to TA thiophene sulfoxide would then occur at the very reactive position 5 of the thiophene ring, leading to a 5-hydroxydihydrothiophene sulfoxide, which should give 5-OHTA by loss of H_2O. Easy dehydration of dihydrothiophene sulfoxides into the corresponding thiophenes has been reported [Mansuy et al. (1991) and references therein]. Alternatively, rather than water, a nucleophilic group of an amino acid residue of the active site could react with TA thiophene sulfoxide in a Michael-type addition reaction. Such a reaction would eventually result in the covalent binding of TA to the P450 2C9/10 active site through an amino acid O(or N or S)–C_5 (thiophene) bond (Figure 11). The immunoblot detection of alkylated P450 2C9 in yeast microsomes incubated with TA and NADPH as the only protein recognized by an antibody raised in rabbits against TA covalently bound to bovine serum albumin by its thiophene 5-position (S. Lecoeur, E. Bonierbale, D. Challine, J. C. Gauthier, P. Valadon, P. M. Dansette, R. Catinot, F. Ballet, D. Mansuy, and P. Beaune, submitted for publication) is in good agreement with this proposition.

The occurrence of covalent binding of TA metabolites to microsomal protein (upon TA oxidation by transformed yeast

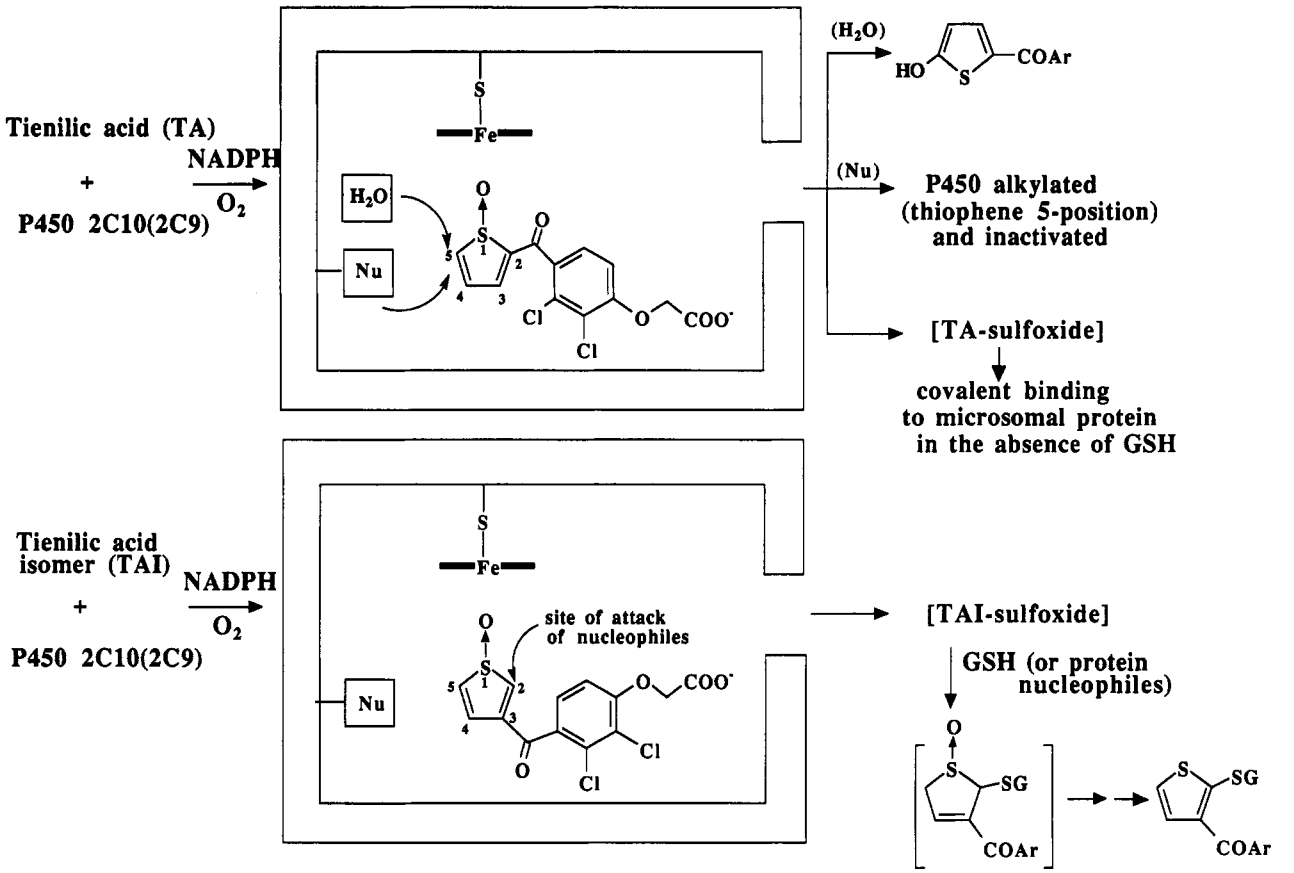


FIGURE 11: Proposed mechanism for the suicide inactivation of P450 2C10(2C9) during the metabolic oxidation of TA: comparison with the oxidation of TAI.

microsomes), which is significantly higher in the absence of GSH than in its presence (Figure 2B), indicates that TA thiophene sulfoxide can diffuse out from the P450 active site and react either with microsomal protein or with GSH present in the medium. The partition ratio of 11–12 determined above means that the competition between H_2O and the active site nucleophile for reaction with TA sulfoxide favors H_2O.

Oxidation of TAI by P450 2C9/10 enzymes leads to an electrophilic thiophene sulfoxide that was shown to react with thiol nucleophiles at position 2 of its thiophene ring (Mansuy et al., 1991) and to bind covalently to protein (Figure 2C). The lack of a mechanism-based inhibitory effect observed with TAI could be due to the different sites of TAI and TA sulfoxides that are attacked by nucleophiles (positions 2 and 5 of the thiophene ring, respectively). The nucleophile of the P450 active site that is responsible for the attack of TA sulfoxide at position 5 may not be positioned to attack TAI at position 2 (Figure 11). TAI sulfoxide would then diffuse out from the enzyme active site and react with GSH or protein nucleophiles in the medium.

It would be also important to know the relevance of these results for what is occurring *in vivo* in humans. Preliminary data concerning the study of TA oxidation by human liver microsomes showed that significant loss of P450 monooxygenase activity (toward 5-hydroxylation of TA), which was dependent on TA activation (with a $t_{1/2}$ of 14 min for 100 μM TA), did exist (data not shown). These results are understandable if one considers that P450 2C9 is one of the major P450 2Cs present in the human liver (Furuya et al., 1991; Guengerich & Turvy, 1991) and seems primarily responsible for TA oxidation by human liver microsomes (López-Garcia et al., 1993). However, complementary experiments using other human liver P450s of the 2C subfamily are necessary to identify definitively all of the human liver P450s involved in TA oxidation and to determine which are inactivated during this oxidation. In this regard, it is interesting to note that P450 2C10 and P450 2C9, which only differ by two amino acids (Umbenhauer et al., 1987; Ged et al., 1988; Brian et al., 1989b), exhibited identical behavior toward the 5-hydroxylation of TA and the P450 inactivation that occurs during this reaction [López-Garcia et al. (1993) and this study]. Also, considering the efficiency of the mechanism-based inactivation of P450 2C10 (2C9) by TA (k_{inact} = 3.6 × 10^{-3} s^{-1}, K_I = 4.3 μM, and partition ratio = 12), as well as the plasma concentrations found upon administration of therapeutic TA doses to human beings (maximal levels of 75 μM to 300 μM after a single oral dose; Vinay et al., 1980), it is likely that suicide destruction of P450 2C9 (2C10) may have occurred *in vivo* upon human exposure to TA. This P450 2C9 (2C10) inactivation by covalent binding of a TA metabolite to the P450 active site should therefore be considered in relation to the appearance of anti-P450 2C (anti-LKM_2) autoantibodies in patients treated with TA and suffering from immunoallergic hepatitis (Beaune et al., 1987). It is known that suicide inactivation of P450 enzymes is followed in some instances by rebound induction of the same P450 (Ortiz de Montellano, 1988). If a similar inductive response occurred in human liver as a consequence of P450 2C9 (2C10) inactivation upon the continued administration of TA, an increase in the level of the target antigen (for anti-LKM2 production and P450 2C recognition) would be expected. Specific alkylation and inactivation of P450 2C9 by TA could therefore be critical events in initiating TA-induced immunoallergic hepatitis.

This study reports for the first time that thiophene derivatives can act as mechanism-based inhibitors of cytochromes P-450. The structural properties of TA make this molecule a good suicide substrate of P450 2C9 and 2C10. Its site of covalent binding in the P450 2C9/10 active site is now under study. This should be very helpful for the determination of the topology of the active site region of this(these) P450 isoenzyme-(s).

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