3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine Photolabels a Substrate-Binding Site of Rat Hepatic Cytochrome P-450 Form PB-4†

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ABSTRACT: Hepatic microsomes isolated from untreated male rats or from rats pretreated with phenobarbital (PB) or 3-methylcholanthrene (3-MC) were labeled with the hydrophobic, photoactivated reagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID). [^{125}I]TID incorporation into 3-MC- and PB-induced liver microsomal protein was enhanced 5- and 8-fold, respectively, relative to the incorporation of [^{125}I]TID into uninduced liver microsomes. The major hepatic microsomal cytochrome P-450 forms inducible by PB and 3-MC, respectively designated P-450s PB-4 and BNF-B, were shown to be the principal polypeptides labeled by [^{125}I]TID in the correspondingly induced microsomes. Trypsin cleavage of [^{125}I]TID-labeled microsomal P-450 PB-4 yielded several radiolabeled fragments, with a single labeled peptide of M_r ~4000 resistant to extensive proteolytic digestion. The following experiments suggested that TID binds to the substrate-binding site of P-450 PB-4. [^{125}I]TID incorporation into microsomal P-450 PB-4 was inhibited in a dose-dependent manner by the P-450 PB-4 substrate benzphetamine. In the absence of photoactivation, TID inhibited competitively about 80% of the cytochrome P-450-dependent 7-ethoxycoumarin O-deethylation catalyzed by PB-induced microsomes with a K_i of 10 μM; TID was a markedly less effective inhibitor of the corresponding activity catalyzed by microsomes isolated from uninduced or β-naphthoflavone-induced livers. P-450 PB-4 dependent microsomal androst-4-ene-3,17-dione 16β-hydroxylase activity was also sensitive to inhibition by TID (IC_{50} ~ 5 μM at 25 μM steroid substrate), with lower sensitivities observed in the case of microsomal androst-4-ene-3,17-dione 6β-hydroxylase (IC_{50} ~ 20 μM), 16α-hydroxylase (IC_{50} > 100 μM), or 7α-hydroxylase (IC_{50} > 100 μM) activities, respectively catalyzed by cytochromes P-450 2a, 2c, and 3. TID inhibited 7-ethoxycoumarin O-deethylation catalyzed by purified and reconstituted P-450 PB-4 with kinetics consistent with mixed inhibition and a K_i of 2 μM. These findings indicate that TID is an active site directed inhibitor of this heme protein and suggest that [^{125}I]TID may serve as a useful probe for the substrate binding site of P-450 PB-4 and perhaps other cytochrome P-450 enzymes found in rat hepatic tissue.

Mammalian liver cytochromes P-450 (P-450)[1] catalyze the oxidative metabolism of structurally diverse lipophilic compounds including many drugs, insecticides, and carcinogens as well as endogenous steroids and fatty acids (Coon & Koop, 1983; Waterman & Estabrook, 1983). At least a dozen or so distinct P-450 forms active in the metabolism of foreign compounds have been purified from livers of untreated rats or from rats pretreated with various monooxygenase inducers including phenobarbital, β-naphthoflavone, isosafrole, and pregnenolone 16α-carbonitrile (Elshourbagy & Guzelian, 1980; Guengerich et al., 1982; Jannson et al., 1985; Ryan et al., 1982a, 1985; Waxman et al., 1983, 1985) with a similar number of P-450 forms also purified from rabbit liver [e.g., see Koop et al. (1981), Dieter & Johnson (1982), and Komori et al. (1984)]. These P-450 forms are structurally distinct, exhibit their own characteristic responses to environmental as well as endogenous factors, and display unique (although often broad) specificity profiles when assayed with a variety of xenobiotic substrates after reconstitution with lipid and NADPH-P-450 reductase.

Although primary structures for several major xenobiotic-inducible rat and rabbit hepatic P-450s have been deduced by a combination of protein and nucleic acid sequencing [reviewed by Black & Coon (1986)], little information is currently available on the identification of the intramembranous region(s) of these integral membrane proteins. Although models have been proposed on the basis of primary sequence data suggesting that the major phenobarbital-inducible P-450 enzymes are embedded in the endoplasmic reticulum by up to eight transmembrane segments (Heinemann & Ozols, 1982; Tarr et al., 1983), hydropathy calculations suggest that of these proposed hydrophobic segments, only the one that is most NH_2-terminal (i.e., residues 3–20 of rat P-450 form PB-4)[2] displays the high degree of hydrophobicity characteristic of transmembrane segments of integral membrane proteins (Waxman & Walsh, 1982; Frey et al., 1985).

In addition to having a lipophilic intramembranous segment(s), mammalian hepatic P-450s are believed to have lipophilic binding sites to accommodate the structurally diverse

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[1] Abbreviations: P-450, cytochrome P-450; PB, phenobarbital; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; SDS, sodium dodecyl sulfate; androstenedione, androst-4-ene-3,17-dione; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] See Materials and Methods for a description of the nomenclature used to describe the individual rat hepatic P-450 forms referred to in this study.

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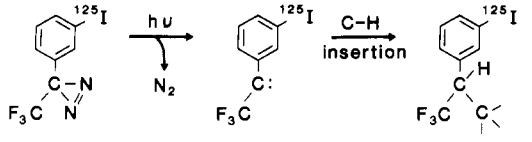
Scheme I



hydrophobic compounds that can serve as substrates. Relatively little is known, however, about the amino acid segments that comprise these substrate binding sites. Biophysical studies indicate the presence of a thiolate as the axial heme ligand of these cytochromes (White & Coon, 1980), and chemical modification studies have suggested the presence of an essential tyrosine at the active site in the case of rabbit liver P-450 form LM2 (Janig et al., 1984). Studies using photoactivated derivatives of the active site heme ligand *N*-phenylimidazole have led to the isolation of labeled heme peptides for several of these proteins (Dus, 1982), while studies with anti-peptide antibodies have suggested the importance of specific peptides for substrate metabolism (Frey et al., 1985). Although suicide inactivators have yielded significant information on the orientation of substrate relative to the P-450 heme iron (Kunze et al., 1983), they have yet to provide information on the specific amino acid residues comprising the substrate binding site.

In this paper we describe the labeling of microsomal P-450 species utilizing a photoactivatable hydrophobic reagent, TID. In previous studies [^{125}I]TID has been shown to react specifically with those segments of membranous polypeptides that are embedded in the phospholipid bilayer (Brunner & Semenza, 1981; Frielle et al., 1982; Brunner et al., 1983). [^{125}I]TID photolabeling of membrane proteins has generally been studied in reconstituted systems using purified enzymes and probably occurs by insertion of a highly reactive carbene into suitably reactive groups, including relatively inert C–H bonds (Scheme I). The covalent TID-amino acid derivatives thus formed are typically stable to conditions required for peptide isolation and sequence analysis (Staros, 1980; Brunner, 1981). Recent reports suggest that the TID-derived carbene may exhibit some preferential labeling of select amino acid residues within transmembrane peptides (Hoppe et al., 1984; Brunner et al., 1985; Meister et al., 1985). Soluble proteins or extramembranous domains of membrane proteins may also be labeled with TID in cases where the tertiary structure of the protein provides for a suitable hydrophobic microenvironment. Thus, calmodulin, a soluble cytoplasmic protein, can be labeled with [^{125}I]TID in the Ca^{2+}-bound conformation only (Krebs et al., 1984). The results presented in this study strongly suggest that [^{125}I]TID labels P-450 PB-4, the major phenobarbital-inducible P-450 enzyme of rat liver microsomes, at a site involved in substrate binding.

Materials and Methods

Materials. Sodium [^{125}I]iodide (carrier-free, 17 Ci/mg) was from New England Nuclear (Boston, MA) and was diluted with unlabeled NaI to a specific activity of about 10 Ci/mmol prior to use. 3-(Trifluoromethyl)-3-[*m*-(formylamino)phenyl]diazirine (diazirine A) was kindly provided by Dr. Josef Brunner (Department fur Biochemie II, ETH, Zurich, Switzerland), and both [^{125}I]TID and nonradioactive TID were synthesized from this precursor as described (Brunner & Semenza, 1981). The final product was purified by thin-layer chromatography on silica gel plates developed with hexane [R_f(TID) 0.7) and then eluted from the silica gel with methanol. The reagent was stable for at least several weeks when stored in methanol in the dark at −20 °C as assessed by silica gel thin-layer chromatography. TID concentrations were determined by UV–visible spectroscopy using a molar extinction coefficient (ethanol) of 300 (Brunner & Semenza, 1981).

Animals and Cell Fractionation Procedures. Rats were induced with PB, 3-methylcholanthrene, or β-naphthoflavone as described previously (Okada et al., 1982; Waxman, 1984) and were fasted for 12 h prior to sacrifice by cervical dislocation. Male white rabbits (New Zealand strain) were used for preparation of anti-P-450 PB-4 heterosera essentially as described (Waxman & Walsh, 1983). Liver microsomes used for the [^{125}I]TID-labeling experiments were isolated from 60-g male rats of the Long-Evans strain (Blue Spruce Farms, Altamount, NY) and were prepared at 4 °C in the presence of 0.1 mM phenylmethanesulfonyl fluoride (added from an 0.1 M stock in 100% ethanol immediately before use) according to Lewis and Sabatini (1977), except that the microsomes were washed by downward-flow chromatography on a column of Sepharose 2B (2.5 × 35 cm) equilibrated in 0.25 M sucrose. Liver microsomal subfractions were prepared as described by Amar-Costesec et al. (1974). Liver microsomal used for catalytic assays were prepared from induced or untreated adult male Sprague-Dawley rats (Charles River Breeding Labs, Inc., Wilmington, MA) as described previously (Waxman, 1984).

P-450 Enzyme Purification and Nomenclature. P-450 PB-4 [also termed P-450 PB-B (Guengerich et al., 1982) or P-450b (Ryan et al., 1982a)] and NADPH-P-450 reductase were purified to apparent protein homogeneity from PB-induced male Sprague-Dawley rats as reported previously (Waxman & Walsh, 1982). P-450 BNF-B [also termed P-450c (Ryan et al., 1982a)] refers to the major hepatic P-450 form induced by treatment of rats with β-naphthoflavone or 3-methylcholanthrene. P-450 enzymes 2a, 2c, and 3 are those described by this laboratory previously (Waxman et al., 1983; Waxman, 1984). Designations given by other investigators to corresponding P-450 forms are detailed in Waxman et al. (1985) and Waxman (1986).

[^{125}I]TID Photolabeling of Hepatic Microsomes. Photoactivation of [^{125}I]TID was accomplished with a 200-W medium-pressure mercury lamp (Hanovia Model 679A36) which was cooled with tap water and shielded with a quartz filter to absorb wavelengths shorter than 315 nm. [^{125}I]TID (7.8 nmol, 120 × 10^6 cpm) was added to liver microsomes (0.5 or 1 mg of protein) suspended in 25 mM sodium phosphate, pH 7.5 (0.5 mL), and placed in a quartz cuvette (15.6 μM [^{125}I]TID, final concentration). Samples were equilibrated on ice for 5 min and then irradiated for 60 s at a distance of 2 cm from the light source. Samples were acetone-precipitated and then dissolved in 1% Triton X-100 for determination of [^{125}I]TID incorporation into protein essentially as described by Frielle et al. (1982). This acetone/Triton extraction procedure was repeated twice (with a 95% recovery of microsomal protein) to remove ^{125}I-labeled microsomal lipids effectively. Aliquots were then subject to SDS-gradient gel electrophoresis or to immunoprecipitation with anti-P-450 PB-4 antibodies followed by SDS-gradient gel electrophoresis and autoradiography.

TID Inhibition of Microsomal Monooxygenase Activities. Liver microsomes (40 μg of protein) isolated from either induced or untreated rats were added to assay buffer A [0.1 M KP_i (pH 7.4), 20% glycerol (v/v), and 0.1 mM ethylenediaminetetraacetate] containing 7-ethoxycoumarin (55 μM to 1 mM) (Aldrich), TID (0–170 μM), and methanol (2.5% v/v) (final concentrations as indicated). Samples were warmed to

37 °C (4 min), and NADPH was then added to give 0.4 mL and 1.0 mM. 7-Hydroxycoumarin formation was determined fluorometrically after a 10-min incubation as described previously (Waxman & Walsh, 1982). In control experiments, TID was shown to have no effect on the fluorescence excitation or emission spectra or fluorescence yield of the 7-hydroxycoumarin product.

For experiments involving TID inhibition of liver microsomal androstenedione hydroxylase activities, liver microsomes (40 μg of protein) diluted into assay buffer B [0.1 M KP_i (pH 7.4) and 0.1 mM ethylenediaminetetraacetate] were added to tubes containing 10 nmol of [4-^{14}C]androstenedione (Amersham; 8 mCi/mmol) and variable amounts of TID dissolved in 6 μL methanol [1.5% (v/v), final methanol concentration]. Reactions were initiated with NADPH and then incubated for 10 min at 37 °C as described earlier for the microsomal 7-ethoxycoumarin metabolism assays. Radiolabeled metabolites were then extracted, chromatographed on silica gel plates, and quantitated by scintillation counting (Waxman et al., 1983; Waxman, 1984).

TID Inhibition of Reconstituted P-450 PB-4 Activity. Purified P-450 PB-4 (12 pmol) was reconstituted with saturating amounts of purified NADPH-P-450 reductase (~35 pmol) and dilauroylphosphatidylcholine clarified by sonication (0.02 μg) in a volume of 0.04 mL for about 10 min at 20–22 °C. Samples were then diluted into assay buffer A containing sufficient substrate and inhibitor to give 67 μM to 1 mM 7-ethoxycoumarin, 0, 5.6, 11.2, 17, or 34 μM TID, and 2.5% methanol (v/v) in the final assay mixture. Samples were warmed to 37 °C (4 min) and assayed for 7-ethoxycoumarin O-deethylation as described above for the microsomal samples except that the NADPH concentration was reduced to 0.3 mM. Lineweaver–Burk plots of the data obtained in the absence of inhibitor indicated a K_m of 0.48 ± 0.06 mM and a V_{max} of 8.9 ± 1.0 nmol of 7-hydroxycoumarin formed min^{-1} (nmol of P-450 PB-4)$^{-1}$. Dixon plots of velocity^{-1} vs. concentration of TID were evaluated by least-squares analyses performed by using (velocity)^2 as a weighting factor as described previously (Waxman & Walsh, 1983). These analyses indicated that five of the six TID inhibition curves intersected a horizontal line drawn at $1/V_{max}$ at a common point whose absolute x value (1.8 ± 0.1 μM) corresponds to the K_i for TID; the sixth curve intersected the horizontal line at an absolute x value of 2.6 μM. A secondary plot of [7-ethoxycoumarin]/K_m + 1 vs. the absolute x intercept of each of the six curves yielded a straight line with a slope = K_i(TID) = 2.4 ± 0.2 μM.

Other Procedures. SDS-gradient (6–11%) polyacrylamide gels were run as described (Laemmli, 1970), and protein was measured by a modification (Markwell et al., 1978) of the method of Lowry et al. (1951) using bovine serum albumin as a standard. Immunoprecipitation of radiolabeled proteins was performed as described (Goldman & Blobel, 1978) with anti-P-450 PB-4 antibodies that were affinity-purified as reported previously (Frey et al., 1985). Immunoprecipitates were analyzed on SDS gels followed by autoradiography using a Cronex intensifying screen and Cronex X-ray film (Du Pont Photo Products, Wilmington, DE). Radioactivity incorporated into specific polypeptides was quantitated by aligning the exposed X-ray film over the dried gel and then cutting out the labeled bands followed by γ counting. NADPH-P-450 reductase was assayed by monitoring reduction of cytochrome c at 550 nm (Waxman et al., 1985).

Results

[^{125}I]TID Incorporation into Microsomal Proteins. Rat hepatic microsomes were photolabeled with [^{125}I]TID followed by the extraction of ^{125}I-labeled lipids and quantitation of ^{125}I incorporation into microsomal proteins (Table I). ^{125}I incorporation into PB-induced hepatic microsomal protein was found to be about 8 times higher than the incorporation into uninduced microsomal protein, while the incorporation of label into microsomal protein from 3-methylcholanthrene-treated animals was about 5 times higher.

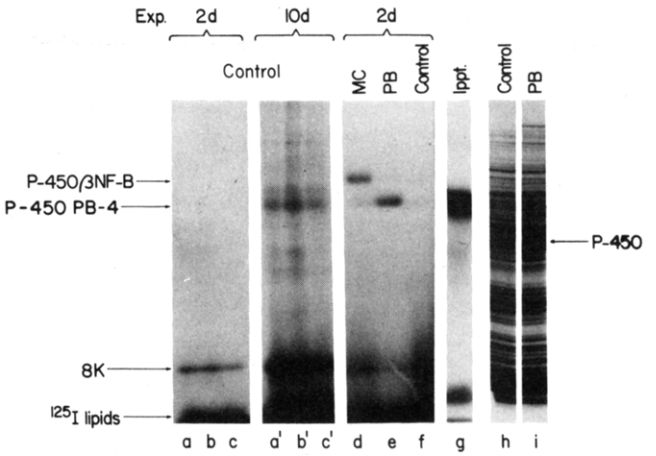


FIGURE 1: SDS gel analysis of [^{125}I]TID-labeled rat liver microsomes. Rat liver microsomes were photolabeled with [^{125}I]TID and then analyzed by SDS-gradient gel electrophoresis and autoradiography (lanes a–g) without extraction of the ^{125}I-labeled lipids. (Lanes a–c and a′–c′) [^{125}I]TID incorporation into uninduced rat liver microsomal subfractions exhibiting varying densities and prepared by isopycnic centrifugation. (Lanes a and a′) [^{125}I]TID-labeled smooth microsomal membranes, density = 1.10; (lanes b and b′) light rough microsomes, density = 1.15; (lanes c and c′) rough microsomes, density = 1.25. (Lanes d–f) [^{125}I]TID labeling pattern of 3-methylcholanthrene-induced ("MC"), PB-induced, and uninduced ("control") liver microsomes, respectively. (Lane g) Immunoprecipitate obtained from an [^{125}I]TID-labeled membrane sample such as the one shown in lane e using 20 μg of affinity-purified anti-P-450 PB-4 IgG; a small contaminant of the precipitate by [^{125}I]lipid is seen at the bottom of the gel. Microsomes analyzed in lanes a–c were prepared from Wistar rats and those in lanes d–g from Long-Evans rats. Lanes a–f were each loaded with about 10^5 cpm of total radioactivity. (Lanes a–c and d–f) Two-day exposure to the X-ray film; (lanes a′–c′) 10-day exposure. The Coomassie Blue stained SDS gel pattern (7% gels) of rough microsomes (250 μg of protein) isolated from untreated and PB-induced rats are shown in lanes h and i, respectively, with the mobility of P-450 PB-4 indicated by the arrow labeled "P-450". The electrophoretic mobilities of P-450s BNF-B and PB-4 were as indicated on the left.

The [^{125}I]TID-labeled proteins found in rough, light rough, and smooth microsomes isolated from uninduced rat liver were then analyzed on SDS gels as shown in Figure 1 (lanes a–c). One prominent band migrating at about M_r 8000 (8 K) represented the major [^{125}I]TID-labeled protein (about 85% of the total label) in each of these highly purified microsomal subfractions. Several proteins migrating in the molecular weight range of the cytochromes P-450 (M_r ~50 000–60 000) could also be identified along with several other peptides upon longer exposure of the gel to X-ray film (lanes a′, b′, and c′). When microsomes prepared from rats treated with xenobiotics known to induce the synthesis of cytochromes P-450 were photolabeled, the pattern of [^{125}I]TID incorporation changed markedly. In microsomes derived from 3-methylcholanthrene-induced rats, [^{125}I]TID labeled a microsomal band of M_r 56 000 (lane d), which corresponds in molecular weight to P-450 form BNF-B, while [^{125}I]TID labeled a prominent band of M_r 52 000 in microsomes prepared from PB-induced rats (lane e). This latter [^{125}I]TID-labeled protein could be specifically immunoprecipitated from detergent-solubilized, [^{125}I]TID-labeled, PB-induced microsomes with

Table I: Incorporation of [^{125}I]TID into Rat Hepatic Microsomes[a]

	^{125}I radioactivity		
	total added (cpm × 10^{-6})	% of total	
		membrane associated	incorporated into protein[b]
uninduced microsomes	115	92.1	0.9
3-methylcholanthrene-induced microsomes	122	91.4	4.8
PB-induced microsomes	126	94.0	7.1
PB-induced microsomes + benzphetamine[c]	120	89.7	1.9

[a] Liver microsomes (1 mg of protein) isolated from untreated rats or from rats induced with 3-methylcholanthrene or PB were incubated with [^{125}I]TID and then photolyzed as described under Materials and Methods. The microsomes were then pelleted by centrifugation (10 min at 12000*g*) and resuspended in 50 mM Tris-HCl (pH 7.5) and 0.25 M sucrose for determination of the total membrane-associated radioactivity. Samples were then acetone-precipitated and extracted twice with 1% Triton X-100 for quantitation of the ^{125}I incorporation into microsomal protein. This extraction procedure was found to have no significant effect on the intensity or pattern of [^{125}I]TID-labeling of microsomal proteins (data not shown). [b] Under the conditions of this experiment, 1% incorporation corresponds to about 0.078 nmol of ^{125}I/mg of microsomal protein. [c] Samples were preincubated with 1 mM benzphetamine hydrochloride for 5 min at 4 °C prior to equilibration with [^{125}I]TID.

a yield of ≥90% using anti-P-450 PB-4 antibodies (lane g) but could not be immunoprecipitated from [^{125}I]TID-labeled microsomes prepared from 3-methylcholanthrene-treated or untreated rats (data not shown). Inclusion of 25 mM glutathione during photolysis to scavenge for TID-derived carbenes present in the aqueous phase had no effect on the magnitude or specificity of labeling of these microsomal proteins.

In contrast to microsomal P-450s PB-4 and BNF-B, the [^{125}I]TID labeling intensity of the 8K protein was largely unaffected by the xenobiotic inducers employed. This unidentified 8K protein is therefore a noninducible polypeptide which, by virtue of its ability to be photolabeled by [^{125}I]TID, serves as a control against which ^{125}I incorporation into other microsomal proteins can be compared. These studies demonstrate that [^{125}I]TID principally labels only two or three microsomal proteins—even though at least 50 major polypeptides can be identified in SDS gel patterns of both uninduced and xenobiotic-induced rat liver microsomes [lanes h and i; see also Kreibich & Sabatini (1974)]. This selectivity is especially striking if one considers that P-450 PB-4[3] contains about 50% of the [^{125}I]TID incorporated into total microsomal protein, even though this P-450 represents only about 8% of the total protein in PB-induced rat liver microsomes.[4]

Proteolysis of [^{125}I]TID-Labeled Microsomes. In order to investigate the exposure of TID-binding segment(s) of P-450 PB-4 on the surface of the microsomal membrane, PB-induced rat liver microsomes were photolabeled with [^{125}I]TID and then subjected to limited proteolysis. Incubation with trypsin degraded microsomal P-450 PB-4 to a series of [^{125}I]TID-labeled fragments ranging in molecular weight from 35 000 to about 4000 (Figure 2). Of particular note was the appearance of an ^{125}I-labeled band migrating at about M_r 4000 (lanes b and c) which was not degraded under conditions of prolonged digestion, during which all higher molecular weight [^{125}I]TID-labeled fragments were completely digested (not shown). This fragment was produced under conditions in which the intensity of the labeled 8K protein was largely unaffected (Figure 2), suggesting that the ^{125}I-labeled 4K fragment is derived from the TID-binding site of microsomal P-450 PB-4.

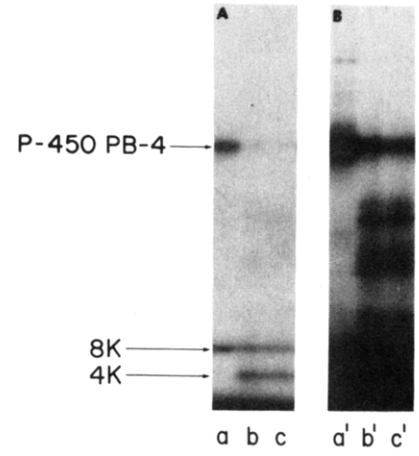


FIGURE 2: Proteolysis of [^{125}I]TID-labeled PB-induced microsomes. [^{125}I]TID-labeled PB-induced microsomes were kept as a control (lanes a and a′) or were treated with trypsin (50 μg/mL) for 10 min at 0 °C (lanes b and b′) or for 10 min at 25 °C (lanes c and c′). SDS (0.8%) and phenylmethanesulfonyl fluoride (1 mM) were then added to the indicated concentrations to stop the reaction. Aliquots containing about 10^5 cpm of radioactivity and corresponding to about 1 μg of protein were analyzed on SDS gels which were then dried and exposed to X-ray film for 2 (panel A) or 10 days (panel B).

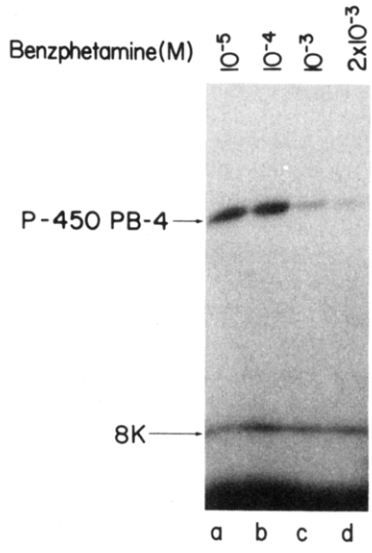


FIGURE 3: Benzphetamine inhibition of [^{125}I]TID incorporation into microsomal P-450 PB-4. PB-induced microsomes were incubated with benzphetamine, photolabeled with [^{125}I]TID, and then analyzed (1 μg of protein/lane) by SDS gel electrophoresis (6–11% gradient gels) and autoradiography. The final concentrations of benzphetamine were as indicated.

Inhibition of [^{125}I]TID Photolabeling of Microsomal P-450 PB-4 by Benzphetamine. The preference by P-450 PB-4 and other cytochromes P-450 for lipophilic substrates suggests the existence of lipophilic residues at the active sites of these integral membrane proteins. In an attempt to distinguish binding of [^{125}I]TID at such a lipophilic substrate-binding site from its binding to a lipophilic intramembranous domain, the

[3] P-450 PB-4 isolated from PB-induced Long-Evans rats migrates slightly more slowly on SDS gels than does P-450 PB-4 from Holtzman or Sprague-Dawley rats (Ryan et al., 1982b; Waxman & Walsh, 1982). Therefore, P-450 PB-4 could not be resolved from the highly homologous P-450 form PB-5 upon SDS gel electrophoresis of these [^{125}I]TID-labeled Long-Evans microsomal proteins, and as such, the major ^{125}I-labeled, PB-inducible M_r 52 000 band may contain a mixture of P-450s PB-4 and PB-5.

[4] Calculation assumes 1.5 nmol of (P-450 PB-4 + P-450 PB-5)/mg of microsomal protein [e.g., see Waxman et al. (1985)] and a molecular weight of 52 000.

Table II: TID Inhibition of 7-Ethoxycoumarin Metabolism Catalyzed by PB-Induced Rat Liver Microsomes[a]

TID (μM)	K_m(apparent) (mM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m
0	0.22 ± 0.08	1.9 ± 0.6	8.9 ± 1.4
5.6	0.40 ± 0.12	1.9 ± 0.5	4.7 ± 0.4
11.2	0.44 ± 0.17	1.9 ± 0.7	4.2 ± 0.4
17	0.55 ± 0.14	1.8 ± 0.5	3.4 ± 0.2
34	0.72 ± 0.29	1.6 ± 0.6	2.2 ± 0.1

[a] PB-induced liver microsomes were assayed for 7-ethoxycoumarin *O*-deethylase activity at seven substrate concentrations (ranging from 55 μM to 1 mM) in the presence of 0–34 μM TID. Shown are the apparent K_m and V_{max} values at each of the indicated TID concentrations, determined by weighted least-squares analysis of Lineweaver–Burk double-reciprocal plots (See Materials and Methods). The increase in apparent K_m without change in V_{max} as a function of increasing TID concentration suggests that TID serves as a competitive inhibitor of this microsomal activity. Enzyme efficiencies are given by V_{max}/K_m (last column). A secondary plot of the slopes of these Lineweaver–Burk double-reciprocal plots (slopes given by K_m/V_{max}) vs. inhibitor concentration yielded a straight line, with a K_i of 10.4 ± 1.2 μM indicated by the absolute value of the x intercept.

effects of the P-450 PB-4 substrate benzphetamine on the [^{125}I]TID photolabeling of microsomal P-450 PB-4 were examined. Incubation of PB-induced microsomes with 1 mM benzphetamine prior to the [^{125}I]TID photolabeling reduced the incorporation of ^{125}I into total membrane protein by about 75% (Table I). SDS gel analysis of the microsomal proteins labeled in the presence of 10 μM to 2 mM benzphetamine indicated that this substrate selectively inhibited the photolabeling of microsomal P-450 PB-4 (Figure 3). ^{125}I incorporation into the 8K peptide was essentially unaffected under conditions where labeling of P-450 PB-4 was completely inhibited. Moreover, [^{125}I]TID incorporation into P-450 BNF-B was only slightly reduced upon preincubation of the 3-methylcholanthrene-induced microsomes with 1 mM benzphetamine (data not shown); this compound is a much less effective substrate for P-450 BNF-B as compared to P-450 PB-4 [e.g., see Ryan et al. (1979)]. Finally, in control experiments, 2-chloronitrobenzene, a hydrophobic substrate of microsomal glutathione S-transferase (Morgenstern et al., 1982) but not a substrate of P-450 PB-4, was shown to have no effect (at 1 mM) on the extent of [^{125}I]TID labeling of either P-450 PB-4 or the 8K peptide (data not shown). These findings suggest that the inhibition of [^{125}I]TID labeling of P-450 PB-4 by benzphetamine does not reflect a nonspecific displacement of the [^{125}I]TID by a more abundant lipophilic compound.

TID Inhibition of P-450-Catalyzed Microsomal Activities. The high specificity of TID for labeling microsomal P-450 PB-4[3] (Figure 1) together with the reduced [^{125}I]TID labeling of this microsomal P-450 in the presence of benzphetamine (Figure 3) suggested that TID may preferentially bind to and photolabel polypeptide segments of P-450 PB-4 which are part of the substrate binding site. Experiments were therefore performed to evaluate the effectiveness of TID as an inhibitor of P-450-catalyzed microsomal monooxygenase activities. In the absence of photoactivation, TID was found to inhibit about 80% of the P-450-dependent 7-ethoxycoumarin O-deethylation catalyzed by PB-induced hepatic microsomes. Under these assay conditions TID had no effect (±10%) on microsomal NADPH-P-450 reductase activity. At a substrate concentration of 120 μM, 50% inhibition of O-deethylation was observed at about 10 μM TID (Figure 4A). At higher substrate concentrations TID was, however, a much less effective inhibitor (e.g., IC_{50} ~ 75 μM at 1 mM 7-ethoxycoumarin), suggesting that 7-ethoxycoumarin competes with TID for

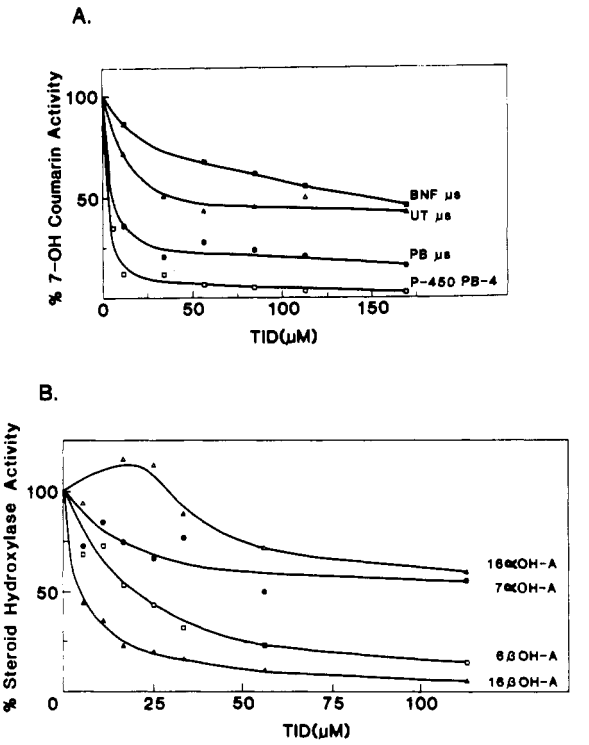


FIGURE 4: TID inhibition of microsomal P-450-dependent monooxygenase activities. (Panel A) Liver microsomes ("μs") isolated from untreated rats ("UT") or rats induced with PB or β-naphthoflavone ("BNF") were assayed for 7-ethoxycoumarin *O*-deethylase activity in the presence of 0–170 μM TID and 0.27, 0.12, and 0.035 mM 7-ethoxycoumarin, respectively, as described under Materials and Methods. [These substrate concentrations are equivalent to half the K_m value for 7-ethoxycoumarin exhibited by each enzyme preparation (determined in the absence of TID).] Under these assay conditions, catalytic activities measured in the absence of TID were 0.28, 0.51, and 2.10 nmol of 7-hydroxycoumarin formed min^{-1} (mg of microsomal protein)$^{-1}$ for the UT, PB, and BNF microsomes, respectively. Purified and reconstituted P-450 PB-4 was assayed at 0.25 mM 7-ethoxycoumarin (K_m ~ 0.5 mM); uninhibited rate = 2.7 nmol of 7-hydroxycoumarin formed min^{-1} (nmol of P-450 PB-4)$^{-1}$. (Panel B) Untreated or phenobarbital-induced rat liver microsomes were assayed for androstenedione hydroxylase activities in the presence of 0–110 μM TID as described under Materials and Methods. Uninhibited catalytic rates corresponded to 0.57, 4.2, and 6.0 nmol of hydroxylated metabolite min^{-1} (mg of microsomal protein)$^{-1}$ for androstenedione 7α-, 6β-, and 16β-hydroxylase activities, respectively (catalyzed by phenobarbital-induced rat liver microsomes), and 1.1 nmol of 16α-hydroxyandrostenedione min^{-1} (mg of microsomal protein)$^{-1}$ (catalyzed by uninduced rat liver microsomes).

binding to the microsomal P-450 enzymes active in its metabolism. More detailed kinetic analyses confirmed that TID serves as a competitive inhibitor of this microsomal activity, with a K_i = 10.4 ± 1.2 μM (Table II). Under comparable assay conditions, TID was a less effective inhibitor of 7-ethoxycoumarin metabolism catalyzed by uninduced or β-naphthoflavone-induced liver microsomes as compared to PB-induced microsomes (Figure 4A). Again, TID was less inhibitory at higher substrate concentrations (e.g., ≤10% inhibition by 170 μM TID of 7-ethoxycoumarin metabolism catalyzed by β-naphthoflavone-induced microsomes when 1 mM substrate is used).

Androstenedione has been shown to serve as a P-450 form specific substrate, with its major hydroxylated metabolites predominantly formed by specific P-450 enzymes (Waxman, 1984; Waxman et al., 1985). In PB-induced rat liver microsomes, androstenedione 16β-hydroxylation is principally catalyzed by immunoreactive P-450 PB-4, androstenedione 7α-hydroxylation by immunoreactive P-450 3, and androstenedione 6β-hydroxylation by immunoreactive P-450 2a.

Moreover, in adult male uninduced (but not PB-induced) liver microsomes, androstenedione 16α-hydroxylase activity is catalyzed by immunoreactive P-450 2c.[5] Androstenedione was therefore used as a substrate to probe for the relative sensitivity of the four indicated P-450 enzymes to TID inhibition in microsomal systems. The results obtained (Figure 4B) indicate that TID inhibits microsomal P-450 PB-4 dependent androstenedione 16β-hydroxylase activity with an IC_{50} of about 5 μM at 25 μM steroid substrate. Although microsomal androstenedione 6β-hydroxylation was also sensitive to inhibition by TID ($IC_{50} \sim 20$ μM), microsomal androstenedione 16α- and 7α-hydroxylations were much less sensitive under the assay conditions employed ($IC_{50} > 100$ μM).[6] These findings are consistent with the high specificity of TID for microsomal P-450 PB-4 indicated by the photolabeling experiments (Figure 1).

Kinetics of TID Inhibition of Purified P-450 PB-4. Although partial (~80%) inhibition was observed with PB-induced microsomes, 7-ethoxycoumarin O-deethylation catalyzed by purified and reconstituted P-450 PB-4 was found to be fully (>95%) inhibition by TID (Figure 4A). Dixon plots (Dixon, 1953) of velocity^{-1} vs. TID concentrations for a family of TID-inhibition curves (see Materials and Methods) yielded a series of straight lines intersecting at a common point, defining a K_i value of 1.8 ± 0.1 μM for TID inhibition in the reconstituted system (data not shown). A similar K_i value (2.3 ± 0.9 μM) was calculated from these same TID inhibition data from a secondary plot of the slopes of a family of double-reciprocal plots [velocity^{-1} vs. (concentration of 7-ethoxycoumarin)$^{-1}$ at each of five inhibitor concentrations] vs. inhibitor concentration. Although linear, these double-reciprocal plots did not intersect the y axis at a common point, indicating that the kinetics of TID inhibition in the reconstituted P-450 system do not fit the pure competitive model observed for the PB-induced microsomes (Table II). These kinetics were also inconsistent with noncompetitive or uncompetitive inhibition and suggested that TID serves as a mixed inhibitor of purified P-450 PB-4.

Discussion

The photoactivated diazirine TID was originally designed as a carbene-generating reagent to label those domains of membrane proteins which are embedded in the hydrocarbon core of the lipid bilayer (Brunner & Semenza, 1981). This reagent has been successfully used to identify the membrane-embedded regions of glycophorin (Brunner and Semenza, 1981), γ-glutamyl transpeptidase (Frielle et al., 1982), sucrase-isomaltase (Spiess et al., 1982), the F_0 subunit of F_1F_0 ATP synthase (Hoppe et al., 1984), one of the transmembrane domains of bacteriorhodopsin (Brunner et al., 1985), and one of the light-harvesting polypeptides of *Rhodospirillum rubrum* (Meister et al., 1985). The data presented in this report demonstrate that [^{125}I]TID can also be used to photolabel several forms of rat liver cytochrome P-450, membrane-bound enzymes catalyzing the oxidative metabolism of lipophilic substrates. In the case of the PB-inducible P-450 form PB-4, several lines of evidence suggested that TID binds at the cytochrome's substrate binding site. In this regard the labeling of P-450 resembles the modification by [^{125}I]TID of a soluble enzyme, calmodulin, at a hydrophobic site (Krebs et al., 1984).

SDS gel analysis of [^{125}I]TID-photolabeled PB-induced microsomes revealed that P-450 PB-4 was the most prominent ^{125}I-labeled microsomal polypeptide. Similarly, in microsomes derived from 3-methylcholanthrene-treated rats, the predominant P-450 species induced by that chemical, P-450 BNF-B, was labeled by [^{125}I]TID to an extent significantly higher than its molar abundance in the population of membrane-associated polypeptides. The specificity of [^{125}I]TID for microsomal P-450 PB-4 was further demonstrated by the fact that incorporation of radiolabel into P-450 PB-4 was selectively inhibited in a dose-dependent manner by preincubation of the membranes with the P-450 PB-4 substrate benzphetamine. Unactivated TID was able to inhibit 7-ethoxycoumarin metabolism catalyzed by PB-induced liver microsomes competitively and with a K_i of 10 μM. Although only about 80% of the 7-ethoxycoumarin *O*-deethylase activity of these microsomes was inhibitable by TID under the assay conditions employed (presumably a reflection of the multiple P-450 enzymes contributing to this microsomal activity),[7] TID effected essentially complete (≥95%) inhibition of this same activity in a purified, reconstituted system as it also did to P-450 PB-4 catalyzed microsomal androstenedione 16β-hydroxylation. Other forms of microsomal P-450, including the pregnenolone 16α-carbonitrile inducible P-450 2a, were found to be at least somewhat sensitive to TID inhibition. Finally, by use of highly purified P-450 PB-4 in an in vitro reconstituted monooxygenase system, TID was shown to be an effective inhibitor ($K_i = 2$ μM) of 7-ethoxycoumarin metabolism. This K_i value was about 5-fold lower than that observed in the microsomal system (Table II), a finding which may reflect, in part, dissolution of TID into the microsomal membrane.

The high specificity of TID for P-450 PB-4 as well as other hepatic P-450 enzymes probably reflects the hydrophobic nature of the P-450 active site. That a single lipophilic compound is bound by several P-450 enzymes which share only limited (~30%) amino acid sequence homology (Black & Coon, 1986) is not surprising given the fact that these monooxygenase catalysts exhibit broad and partially overlapping substrate specificities. The inhibition of [^{125}I]TID modification of microsomal P-450 PB-4 by benzphetamine and the kinetics of TID inhibition of P-450 PB-4 activity suggest that [^{125}I]TID does not photolabel P-450 PB-4 at its site of interaction with NADPH-P-450 reductase. TID probably does not modify the most likely candidate for a hydrophobic membrane-insertion peptide (P-450 PB-4 residues 3–20; Frey et al., 1985) since it has been demonstrated that the corresponding segment (residues 1–30) is not required for catalytic activity in the case of P-450 BNF-B (Sakaki et al., 1985).

Although TID served as a competitive inhibitor of 7-ethoxycoumarin metabolism catalyzed by PB-induced liver microsomes, mixed inhibition kinetics were observed for purified P-450 PB-4 in a reconstituted system. This suggests that the binding of TID and substrate might not be entirely exclusive in the case of the purified enzyme. Although appropriate steps were taken to prevent photoactivation of the reagent during the course of the inhibition experiments, it is possible that some

[5] These conclusions are largely based on antibody inhibition studies, and as such, the possibility that immunochemically related P-450 enzymes not yet purified may also contribute to these microsomal activities cannot be eliminated. For this same reason it is also unclear whether P-450 PB-5 contributes to microsomal androstenedione 16β-hydroxylation.

[6] Microsomal androstenedione 7α-hydroxylase exhibits a much lower K_m for androstenedione ($K_m \leq 3$ μM) than do the other three microsomal androstenedione hydroxylases ($K_m = 10$–30 μM) (D. J. Waxman, unpublished experiments). Thus, the lower degree of TID inhibition observed for this P-450 3 dependent microsomal activity might not reflect its inherent sensitivity to TID inhibition.

[7] Antibody inhibition experiments suggested that only about 40% of the 7-ethoxycoumarin *O*-deethylase activity of the PB-induced microsomes was catalyzed by immunoreactive P-450 PB-4 at 1 mM substrate (D. J. Waxman, unpublished experiments).

covalent modification of the reconstituted P-450 PB-4 did occur. In any event, the data obtained are consistent with a model whereby TID binds to P-450 PB-4 at or near the substrate binding site and, as such, TID should serve as a useful probe for the identification of active site residues of P-450 PB-4 and perhaps other P-450 enzymes as well. These may include P-450 BNF-B (Figure 1) in addition to P-450 2a, which was also shown to be sensitive to inhibition by TID (Figure 4B). In all likelihood [^{125}I]TID covalently modifies one or more amino acid residues of P-450 PB-4 rather than one of the tetrapyrrole nitrogen atoms of the active site heme, as suggested by the stable association of the ^{125}I label with the protein band under conditions of denaturation and gel electrophoresis in the presence of SDS. Although the precise amino acid residue(s) modified by [^{125}I]TID have yet to be identified, trypsin digestion of ^{125}I-labeled microsomal P-450 PB-4 suggests that they are localized to discrete peptide about 4000 daltons in size.

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Registry No. TID, 81340-56-9; cytochrome P-450, 9035-51-2; steroid 16β-hydroxylase, 37359-58-3; steroid 6β-hydroxylase, 9075-83-6; steroid 16α-hydroxylase, 37364-16-2; steroid 7α-hydroxylase, 39346-35-5; 7-ethoxycoumarin *O*-deethylase, 42613-26-3; androstenedione, 63-05-8; 7-ethoxycoumarin, 31005-02-4.

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