Human Protoporphyrinogen Oxidase: Relation between the Herbicide Binding Site and the Flavin Cofactor†

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ABSTRACT: Protoporphyrinogen IX oxidase (protox) catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX in the penultimate step of heme and chlorophyll biosynthesis in animals and plants. Protox is the target of light-dependent peroxidizing herbicides and is inhibited at nanomolar levels by several chemical classes including tetrahydrophthalimides (discussed below) and diphenyl ethers (e.g., acifluorfen) usually with little selectivity between the mammalian and plant enzymes. The herbicide binding site is examined here with a photoaffinity radioligand optimized on the basis of structure−activity relationships. A radiosynthetic procedure is described for this new herbicidal probe, *N*-(5-azido-4-chloro-2-fluorophenyl)-3,4,5,6-[^{3}H]tetrahydrophthalimide ([^{3}H]AzTHP), resulting in high specific activity (2.6 TBq/mmol). Human protox expressed in *Escherichia coli* and purified by affinity chromatography is used with [^{3}H]AzTHP to characterize the herbicide/substrate binding site. Specific binding of [^{3}H]AzTHP to human protox is rapid, completely reversible in the absence of light with a K_d of 93 nM, and competitively inhibited by the 5-propargyloxy analogue and by acifluorfen, which are known to bind at the substrate (protoporphyrinogen) site. The B_{max} establishes one [^{3}H]AzTHP binding site per FAD. Diphenyleneiodonium, proposed to inhibit protox by interaction with the FAD cofactor, inhibits enzyme activity by 48% at 100 μM without affecting [^{3}H]AzTHP binding in the presence or absence of substrate, suggesting that the herbicide binding site may not be proximal to FAD. The first step has been taken in photoaffinity labeling the herbicide/substrate site with [^{3}H]AzTHP resulting in apparent covalent derivatization of 13% of the herbicide binding site.

Protoporphyrinogen oxidase (protox,[1] EC1.3.3.4) catalyzes the exchange of six hydrogen atoms from protoporphyrinogen IX to oxygen, producing the aromatic heme and chlorophyll precursor protoporphyrin IX and hydrogen peroxide; it is the last common enzyme in heme and chlorophyll biosynthesis. Protox from mammals and yeast contains FAD which probably participates in redox cycling essential to the catalytic mechanism (*1*−*3*). A conserved sequence near the N-terminus is assigned as a putative FAD binding site based on its similarity to other flavin-containing enzymes (*3*). Human protox has been cloned and expressed in *Escherichia coli* with a terminal histidine sequence for purification on nickel affinity matrixes. This expressed enzyme is a homodimer consisting of two 51 kDa subunits but containing only a single FAD per dimer (*3*). The sensitivity of cloned protox from human placenta to acifluorfen (Figure 1) ($IC_{50} \sim 4$ μM) (*3*) is similar to that from placental membrane preparations and other mammalian sources (*4*).

Protox is important as the target of light-dependent peroxidizing herbicides (*5*) and candidate photodynamic therapy anticancer drugs (*5*, *6*) and because a mutation conferring low activity leads to the human hereditary disease variegate porphyria (*7*). The herbicides undergo rapid, reversible specific binding (*8*) and compete with protoporphyrinogen IX for the substrate binding site on protox in plants (*9*, *10*). Kinetic studies with mammals and plants confirm that herbicide inhibition of enzyme activity is competitive with substrate (*11*). Little difference is observed with many herbicidal inhibitors between protox from various mammals and plants (*4*, *8*, *12*, *13*). Protox inhibition in plants causes an accumulation of protoporphyrinogen that is exported into the cytoplasm where it is oxidized to protoporphyrin by herbicide-insensitive oxidizing factors (*14*, *15*). Accumulated protoporphyrin then generates highly reactive oxygen species in the presence of light which induce lipid membrane peroxidation (*16*). Similar mechanisms of phototoxicity are proposed for photodynamic therapy (*5*, *6*) and variegate porphyria (*17*). In contrast, although not known to be an herbicide, diphenyleneiodonium (DPI) also inhibits protox, exhibiting essentially irreversible slow-

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[1] Abbreviations: AzTHP, *N*-(5-azido-4-chloro-2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide; [^{3}H]AzTHP, *N*-(5-azido-4-chloro-2-fluorophenyl)-3,4,5,6-[^{3}H]tetrahydrophthalimide; B_{max}, maximum binding capacity; BOG, *n*-octyl-β-D-glucopyranoside; DPI, diphenyleneiodonium; LSC, liquid scintillation counting; protox, protoporphyrinogen IX oxidase; TFA, trifluoroacetic acid; THP, *N*-[4-chloro-2-fluoro-5-(propargyloxy)phenyl]-3,4,5,6-tetrahydrophthalimide; [^{3}H]THP, *N*-[4-chloro-2-fluoro-5-(propargyloxy)phenyl]-3,4,5,6-[^{3}H]tetrahydrophthalimide.

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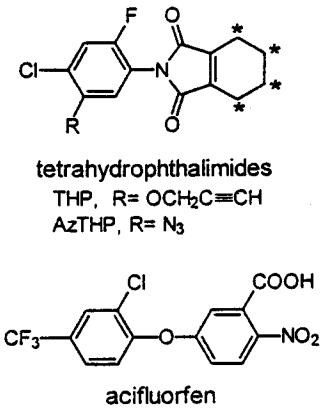

FIGURE 1: Protox inhibitors studied. Asterisks designate positions of tritium in [^{3}H]THP and [^{3}H]AzTHP.

binding kinetics probably associated with formation of a covalent adduct to reduced FAD (*18*).

Two general classes of herbicidal protox inhibitors are the aryl heterocycles (e.g., *N*-[4-chloro-2-fluoro-5-(propargyloxy)phenyl]-3,4,5,6-tetrahydrophthalimide or THP) and the diphenyl ethers (e.g., acifluorfen) (Figure 1). They have contributed importantly in understanding the structure, mechanism, and function of protox. Tritiated THP ([^{3}H]THP) binds with high affinity to a single saturable site in protox from solubilized mouse liver mitochondria and is displaced by the diphenyl ether oxyfluorfen in vivo (*8*). Two types of photoaffinity probes have been prepared previously for protox: THP analogues, such as with a 5-(4-azidobenzyl) substituent, which were not radiolabeled for biochemical studies (*19*), and the diazoketone analogue of [^{3}H]acifluorfen which binds to a 55 kDa protein in yeast (*20, 21*). This report describes the development and radiosynthesis of the 5-azido analogue of THP (AzTHP and [^{3}H]AzTHP) (Figure 1) for use in defining the relation between the herbicide binding site and the flavin cofactor.

MATERIALS AND METHODS

Chromatography. Reversed-phase HPLC involved a protein C_4 column (25 × 0.46 cm, 5 μm, Vydac Separations Group, Hesperia, CA) using water (2 min) then water-acetonitrile gradients, with each solvent containing 0.1% trifluoroacetic acid (TFA). The gradient for determining the purity of [^{3}H]AzTHP was 0−35% for 10 min, then 35−60% for 20 min, monitoring 1-min fractions by liquid scintillation counting (LSC) (and also on cochromatography with unlabeled standard at 220 nm) (t_R 25 min). The gradient for analysis of FAD was 0−55% from 2 to 13 min, monitoring at 446 nm (t_R 11 min).

Chemicals and Synthesis. General. AzTHP and [^{3}H]-AzTHP were prepared as shown in Figure 2. The Supporting Information gives full characterization data on the compounds described below. Sources for other chemicals were as previously reported (*8*).

*N-(4-Chloro-2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide (**1**).* A solution of 4-chloro-2-fluoroaniline (4.36 g, 30.0 mmol) and 3,4,5,6-tetrahydrophthalic anhydride (4.65 g, 30.6 mmol) (both from Aldrich Chemical Co., Milwaukee, WI) in glacial acetic acid (16 mL) was refluxed for 5 h. The solution was then cooled and poured into ice. The mixture was extracted with ethyl acetate, dried ($MgSO_4$), filtered, and concentrated in vacuo to give a viscous oil. Flash chromatography (silicic acid developed with 0−20% ethyl acetate in hexane) gave 8.32 g of a white solid (mp 74 °C) in 99% yield.

*N-(4-Chloro-2-fluoro-5-nitrophenyl)-3,4,5,6-tetrahydrophthalimide (**2**).* A solution of **1** (4.20 g, 15.0 mmol) in concentrated H_2SO_4 (98%, 50 mL) was cooled to 0 °C and concentrated HNO_3 (1.41 g, 15.7 mmol) was added slowly. The resulting yellow solution was further stirred for 2 h. Then, it was poured into ice and the resulting precipitate was filtered and washed with cold water to give a cream-colored solid (4.20 g, 92% yield after flash chromatography with silicic acid as above, mp 155−156 °C).

*N-(5-Amino-4-chloro-2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide (**3**).* A solution of the nitroaryl compound (**2**) (2.00 g, 6.16 mmol) and $SnCl_2 \cdot 2H_2O$ (6.95 g, 30.8 mmol) in absolute ethanol (20 mL) and ethyl acetate (20 mL) was heated at 70 °C for 1 h. It was then cooled and poured into ice-cold water. A saturated solution of $NaHCO_3$ was added afterward, and the mixture was extracted with ethyl acetate, dried ($MgSO_4$), filtered, and concentrated in vacuo to give 1.72 g of a yellowish solid (mp 160−161 °C) in 95% yield.

N-(5-Azido-4-chloro-2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide (AzTHP). To amine **3** (1.18 g, 4.0 mmol) in ice-cold concentrated HCl (12 N, 10 mL) was added a solution of $NaNO_2$ (0.83 g, 12.0 mmol) in ice-cold water (5 mL). After the mixture was stirred at 0 °C for 30 min, a solution of NaN_3 (1.30 g, 20.0 mmol) in ice-cold water (5 mL) was added dropwise, and the mixture was stirred for an additional 2 h. Ether was then added and the organic phase was separated and washed with $NaHCO_3$, dried ($MgSO_4$), filtered, and concentrated in vacuo. Flash chromatography purification on silicic acid using 10−20% ethyl acetate in hexane gave 1.25 g of a cream-colored solid (mp 140 °C) in 97% yield. λ 229, ϵ = 3900; λ 226, ϵ = 29000 (absolute ethanol).

*5-Azido-4-chloro-2-fluoroaniline (**4**).* AzTHP (160 mg, 0.50 mmol) was dissolved in a minimal amount of CH_2Cl_2 and then diluted in methanol (15 mL) followed by slow addition of ammonium hydroxide solution (58%, 7.5 mL). The resulting solution was stirred at 25 °C for an additional 5 min. Then, the solution was concentrated in vacuo and the residue was purified by flash chromatography as above using methylene chloride to give 88 mg of a yellowish solid (mp 75−76 °C) in 94% yield. λ 314, ϵ = 4000; λ 232, ϵ = 19500 (absolute ethanol).

[^{3}H]AzTHP. The procedures for synthesis and assignment of structure and specific activity were similar to those in our report on [^{3}H]THP (*8*). Freshly prepared [^{3}H]3,4,5,6-tetrahydrophthalic anhydride (10 mg) was treated with azidoaniline **4** (13 mg) in glacial acetic acid (0.5 mL) in minimal light followed by refluxing for 4 h. The residue after lyophilizing overnight was dissolved in freshly distilled THF and chromatographed by normal-phase HPLC as before (*8*) except using hexane-THF (92.5:7.5 for 8 min then 85:15 rinse) (yield 24% based on UV absorbance). Final purification was on silicic acid in a pasteur pipet introducing [^{3}H]AzTHP in hexane and eluting with hexane:ethyl acetate 20:1, then 10:1. Radiochemical purity was >98% based on reversed-phase HPLC, and the specific activity was 2.6 TBq/mmol.

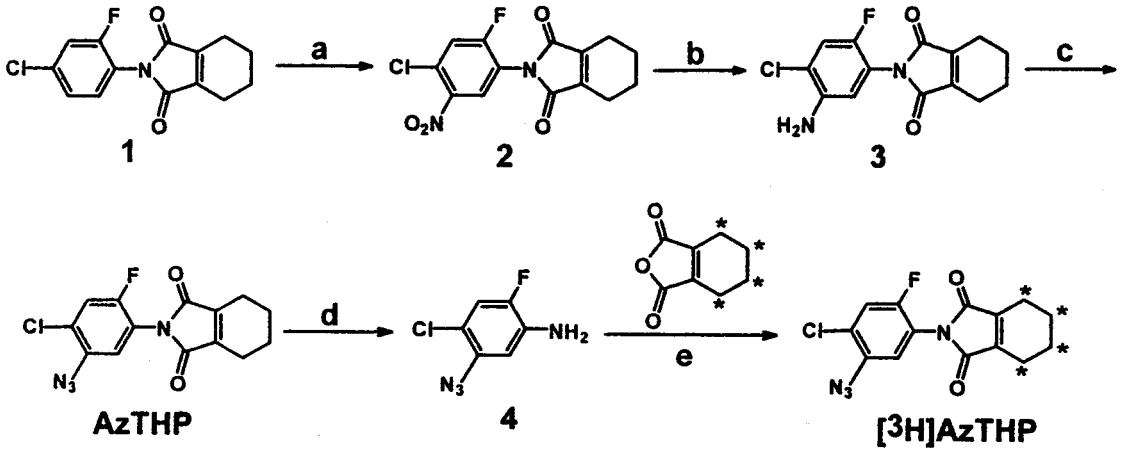

FIGURE 2: Synthesis of AzTHP and [^{3}H]AzTHP. (a) HNO_3, H_2SO_4, 92%; (b) $SnCl_2 \cdot 2H_2O$, C_2H_5OH:$C_2H_5OC(O)CH_3$, 95%; (c) HCl, $NaNO_2$, NaN_3, H_2O, 0 °C, 97%; (d) NH_4OH (58%), CH_2Cl_2, CH_3OH, 94%; (e) $CH_3C(O)OH$, reflux, 24%.

Herbicidal Activity. The potencies of THP and AzTHP were compared with velvetleaf (*Abutilon theophrasti*) cotyledons on 4−5 cm tall plants (provided by R. J. Anderson, Novartis Crop Protection, Palo Alto, CA) by application of the test compound in acetone:ethanol 1:1 (1 μL) as a single spot on the upper surface. The plants were held in the dark for 3 h (to allow penetration of THP and the photolabile AzTHP), then exposed to normal ambient sunlight for 4 h and the following day, with comparable controls maintained in the dark. Ratings were made on the basis of leaf burn.

Human Protox and FAD Content. Plasmid pHPPO-X (containing the sequence for human placental protox with an attached histidine sequence for affinity purification) was used to transform *E. coli* (JM109) (*3*). Rich broth (150 mL) (Circlegrow, BIO101 Inc., Vista, CA) containing ampicillin (25 μg/mL) was inoculated with a single colony followed by incubation and shaking at 250 rpm for 20 h at 30 °C, then using this culture to inoculate 1 L of the same broth followed by 20 h growth as above (*22*). The pelleted *E. coli* was suspended in solubilization buffer [50 mM phosphate (pH 8.0) containing 0.2% *n*-octyl-β-D-glucopyranoside (BOG) (Sigma, St. Louis, MO) and phenylmethylsulfonyl fluoride (10 μg/mL)]. Next, the BOG concentration was raised to 1% followed by strong sonication then centrifugation at 100000*g*. The clarified extract was stirred with 50% nickel affinity matrix (Talon affinity resin) (3.5 mL) (Clontech Inc., Palo Alto, CA) for 30 min at room temperature, then the matrix was washed in a column with solubilization buffer as above (5 mL), and the same buffer containing 20% ethanol (18 mL) before eluting bound protox with solubilization buffer containing 300 mM imidazole (pH 8.0) (*23*). The eluent contained 4−10 mg protein [measured by the Bradford method with bovine serum albumin as the standard (*24*)] and very high protoporphyrinogen oxidizing activity (230 nmol min^{-1} mg^{-1} protein).

The FAD content of the protox was determined directly by injection of 13 μg protein (*24*) in 0.1% TFA in water, then HPLC analysis against a standard curve for FAD. Recovery values for FAD from protox on filters in binding studies as below were determined by oven drying the filters under mild conditions (90 °C, 2 min), then extraction with 0.1% TFA (0.5 mL) for 15 min and HPLC. FAD recoveries from spiked filters were 93% or greater.

Radioligand Binding. The methodology applied earlier (*8, 13*) for [^{3}H]THP binding in solubilized mouse mitochondrial preparations was used with a few modifications for [^{3}H]-AzTHP binding in human protox. [^{3}H]AzTHP (specific activity adjusted to 8.9 GBq/mmol) was incubated with human protox (13 μg protein) in 100 mM phosphate buffer (pH 7.2) with 0.4% BOG (0.5 mL) for 15 min at 25 °C. The radioligand was at 80 nM in optimization experiments and with DPI and at 62.5−1500 nM for Scatchard plots. $(NH_4)_2SO_4$ (60% saturated, pH 7.2, 0.5 mL) was then added, followed by vortexing the mixture and allowing it to sit 15 min before filtration with a Brandel Model M-24R cell harvester (Gaithersburg, MD) using GF/C filters (prewashed with 10 mM phosphate buffer, pH 7.2) and rinsing three times with this buffer (2 mL) at 5 °C. Filters were then soaked in Optiphase HiSafe 2 scintillation cocktail (Wallac OY, Turku, Finland) (1 mL) prior to LSC. All inhibitors and ligands were added in dimethyl sulfoxide which never exceeded 1.5% and was constant in control and test samples. Experiments with photosensitive compounds were carried out in subdued light. In competitive binding studies, incubations included THP (5 nM) or acifluorfen (500 nM). All concentrations are based on the 1-mL volume after adding the $(NH_4)_2SO_4$ solution because of the continuing association and dissociation during this step. Nonspecific binding was determined with 500 μM acifluorfen at each ligand or inhibitor concentration. FAD recovery on filters was used to measure protox retention in binding assays.

[^{3}H]THP binding assays with mouse protox used liver mitochondrial membranes (solubilized with 1% sodium cholate) and differed from the human protox assays in deleting BOG from the incubation mixture and using the radioligand at 0.5 nM (*8, 13*).

Effect of DPI on [^{3}H]AzTHP Binding and Enzyme Activity of Human Protox. In [^{3}H]AzTHP binding inhibition experiments (performed only with human protox), 0.5−100 μM DPI (Alexis Corp., San Diego, CA) was first incubated with the enzyme in pH 7.2 buffer as above for 15 min. Protoporphyrinogen (5 μM) was then added in buffer (75 μL), followed by a second incubation period of 15 min before addition of $(NH_4)_2SO_4$ and analysis as above. Conditions were the same in assays without substrate except the second incubation period was omitted. For direct comparison to enzyme assays, the effect of DPI on enzyme catalysis was determined under conditions similar to those used in binding assays. Protox (39 μg) was incubated with DPI (100 μM) in phosphate buffer (3 mL) containing Tween 20 (0.5%) for

15 min in clear-walled cuvettes. Protoporphyrinogen (5 μM) was then added and protoporphyrin fluorescene development monitored (λ_{ex}: 405 nm; λ_{cm}: 635 nm) for 2 min. Protoporphyrinogen was prepared by reducing protoporphyrin in 20% ethanolic 10 mM potassium hydroxide and 66 mM sodium ascorbate using 5% sodium−mercury amalgam (*8*).

Protox assays are normally optimized by addition of a reducing agent (usually dithiothreitol) (*13*, *25*) and measurement of protoporphyrin production by spectrofluorometry (*26*). The reducing agent lessens nonenzymatic oxidation of protoporphyrinogen to protoporphyrin, while fluorescence measurements enhance the sensitivity allowing protox catalysis to be measured in tissues at low levels. Assays in the present study of *E. coli*-expressed human enzyme contained sufficiently high concentrations of protox to make enzymatic catalysis in the absence of reducing agents much greater than nonenzymatic oxidation; i.e., background oxidation was 10% relative to uninhibited enzyme.

Photoaffinity Labeling. Human protox (53 μg protein) was incubated with [^{3}H]AzTHP (8.9 GBq/mmol) (1.5 μM) in 100 mM phosphate buffer (pH 7.2) (2 mL) in a clear-sided UV silica cuvette for 15 min, followed by irradiation in a Rayonet Photochemical Reactor (The Southern New England Ultraviolet Co., Middletown, CT) with five 300 nm lights for 10 min; this irradiation time completely photodecomposes the radioligand. The reaction mixtures were then treated with $(NH_4)_2SO_4$, filtered, and washed as above. Specific (protectable) binding was determined with THP (10 μM) instead of acifluorfen because of its lower UV absorbance at inhibitory concentrations. Noncovalent binding was defined as that which could be displaced by acifluorfen after photoreaction. Specific photoaffinity labeling or covalent binding was considered to be that portion protectable by THP on irradiation and not displacable by acifluorfen after irradiation.

RESULTS AND DISCUSSION

Optimization of Photoaffinity Probe. Structure−activity studies were used to optimize the photoaffinity probe for the herbicide site using solubilized mouse liver mitochondria and [^{3}H]THP. High herbicidal potency in the tetrahydrophthalimides is conferred by 4-chloro (or 4-bromo)-2-fluoro substituents (*27*) with a suitable moiety in the 5-position (*28*) so the focus here was on the 4-chloro- or 4-bromo option and selecting an appropriate photoreactive moiety for the 5-substituent. The high potency of the parent compound (**1**) (IC_{50} 20−30 nM) is greatly diminished on nitration and reduction (to give **2** and **3**, IC_{50} ∼400 nM) but restored on conversion to the azido compound (AzTHP, IC_{50} 20−30 nM). Although not detailed here, two other observations are relevant. High potency is also achieved with the 5-(4-*tert*-butylbenzyl) ether (IC_{50} 20−30 nM) which opened the possibility of using the 5-[4-(trifluoromethyldiazirinyl)-benzyl] ether as the photoreactive moiety (*19*, *29*). However, AzTHP was preferred because its photoactivatable moiety is at the core of the molecule and is thus more likely to label the enzyme active site rather than a peripheral location. Further, two 4-bromo compounds were of similar potency to their 4-chloro analogues **1** and AzTHP. Ultimately AzTHP with the chloro substituent was selected for detailed study because it is the closest analogue to the commercial herbicides.

Herbicidal Activity of AzTHP. AzTHP was applied to velvetleaf cotyledons to determine possible herbicidal activity with the very potent THP as the standard. In dark controls, there is no leaf burn at 10 μg/cotyledon. In sunlight, THP causes severe damage at 1 ng/cotyledon and AzTHP gives similar burn at a 10-fold higher dose. On this basis, AzTHP is a suitable model for the light-dependent peroxidizing herbicides.

Synthesis and Reactivity of [^{3}H]AzTHP (Figure 2). The starting material (**1**) (*27*), from coupling two commercially available compounds in acetic acid, is nitrated exclusively in the 5-position with one molar equivalent of HNO_3 in concentrated H_2SO_4. Selective reduction of the nitro compound (**2**) to the amine (**3**) with $SnCl_2 \cdot 2H_2O$ (*30*, *31*), then diazotization gives AzTHP. As shown before for THP (*8*), AzTHP is quickly hydrolyzed at room temperature in NH_4-OH to azidoaniline **4**, providing the required intermediate for coupling to tritium-labeled tetrahydrophthalic anhydride (*8*) by refluxing in acetic acid to give [^{3}H]AzTHP at 2.6 TBq/mmol.

Binding Parameters for [^{3}H]AzTHP with Human Protox. Specific binding is 90−98% of total binding (depending upon the radioligand concentration), and Scatchard analysis reveals a single high-affinity binding site (K_d 93 nM) (Figure 3). The highly potent THP (5 nM) and less effective acifluorfen (500 nM) are competitive inhibitors of [^{3}H]AzTHP binding (Figure 3). In minimal light, binding is totally reversible following equilibration with rapid and complete displacement by acifluorfen (500 μM). The action of protox-inhibiting herbicides is competitive with respect to substrate (*11*). Thus, the herbicide binding site is the same as or overlapping the substrate site so characterizing one may define the other. This is consistent with studies of quantitative structure−activity relationships which suggest a steric complementarity of protox-inhibiting herbicides with one-half of protoporphyrinogen (*32*). It is therefore likely that protox from eukaryotic sources, including humans, contains a shared herbicide-substrate site.

The binding parameters observed with [^{3}H]AzTHP and human protox (Figure 3) justify its selection as the photoaffinity probe from preliminary studies above. The incubation time to reach equilibrium, examined from 2 to 60 min, is 10 min before addition of $(NH_4)_2SO_4$ and filtration 15 min later gives optimal retention on the filters. Recovery of bound [^{3}H]AzTHP is increased from 23% without to 36% with $(NH_4)_2SO_4$. The observed maximal binding capacity (apparent B_{max}) is 4.9 pmol/μg protox protein or 13.7 pmol/μg corrected for 36% recovery which corresponds to 1 pmol AzTHP per 1.4 pmol protox (51 kDa). This ratio for the herbicide binding site:protox monomer is only an approximation because of uncertainties in the Bradford protein assay (*24*) using albumin as a surrogate for protox standard. It is likely that differences in the amino acid composition of protox (normally membrane bound) and albumin (soluble) will affect the proteins' interactions with the Coomassie blue dye of the Bradford assay (*33*) thus changing the relative response of the assay to the two proteins.

Stoichiometry of [^{3}H]AzTHP Binding Site in Human Protox Relative to FAD Content. The strong absorbance of FAD and good chromatographic characteristics were used in two ways in this study. First, FAD recovered on glass fiber filters provided a measure of protox retained after

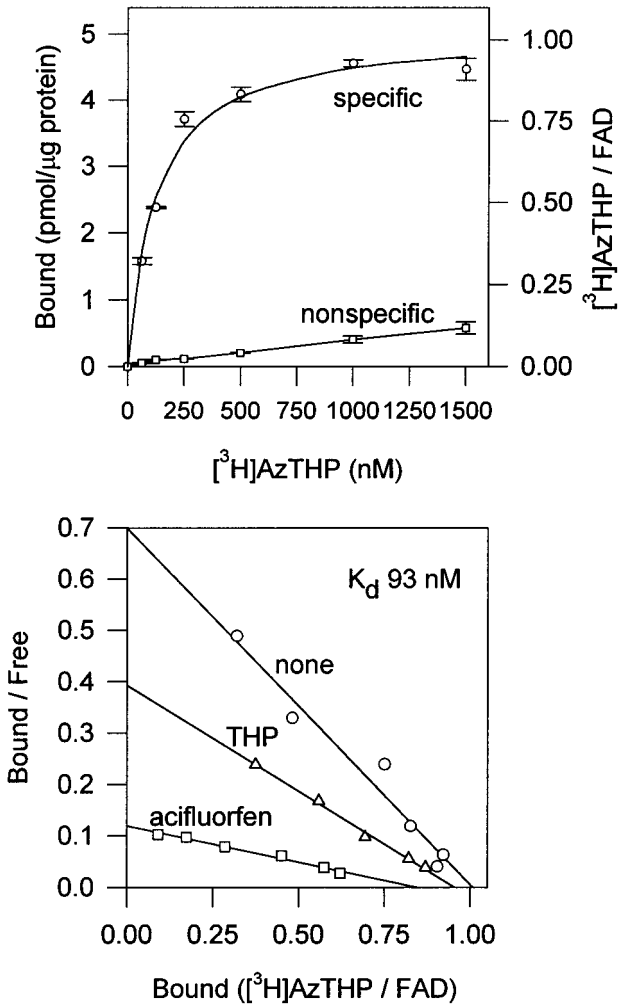

FIGURE 3: Binding parameters for [^{3}H]AzTHP with human protox and Scatchard analysis of inhibitor effects. Protox (13 μg protein) was incubated with [^{3}H]AzTHP (62.5−1500 nM) in 100 mM phosphate buffer at pH 7.2 containing 0.4% BOG (0.5 mL) for 15 min at 25 °C, followed by addition of $(NH_4)_2SO_4$ (60% saturation, pH 7.2, 0.5 mL), vortexing, and filtration after 15 min. THP (5 nM) and acifluorfen (500 nM) were added to the [^{3}H]AzTHP solution before the protein. Nonspecific binding was determined with acifluorfen at 500 μM. Bound [^{3}H]AzTHP is given in pmol/μg protox protein (uncorrected for loss) and in relation to FAD retained on the filter.

filtration showing the recovery of protox on the filters is 36% of that used in incubations. Second, the molar quantity of FAD was compared to [^{3}H]AzTHP binding sites to determine the herbicide binding site:FAD ratio. Radioligand binding at saturation is equal to the FAD content extracted from the filters on a molar basis; i.e., the [^{3}H]AzTHP/FAD ratio is 1:1 (Figure 3). Human protox contains a single FAD cofactor per homodimer protein (*3*), thus protox dimers contain one FAD cofactor and one herbicide binding site.

DPI and [^{3}H]AzTHP Binding in Human Protox. Protox contains FAD as a cofactor which probably assists in the oxidation of protoporphyrinogen. The inhibitor DPI is proposed to be converted by flavin redox cycling to a phenyl radical which derivatizes enzymes at or near their flavin cofactors causing irreversible covalent inhibition (*34*). Consistent with these observations, DPI also inhibits human protox activity by 48% at 100 μM, reducing protox activity to 110 nmol min^{-1} mg^{-1} protein. In contrast to acifluorfen and THP, DPI does not however inhibit [^{3}H]AzTHP binding

Table 1: Photoaffinity Binding Parameters of [^{3}H]AzTHP to *E. coli* Expressed Human Protox[a]

incubation	covalent binding dpm × 10^3	covalent binding %	type of covalent binding
[^{3}H]AzTHP	114	100.0[b]	total
[^{3}H]AzTHP + THP	84	73.7	nonspecific
(difference due to THP)	30	26.3[c]	specific

[a] The experiment has two steps. First, protox (53 μg) was incubated with [^{3}H]AzTHP (1.5 μM) and THP (0 or 10 μM) for 15 min in the dark before exposure to 300 nm light for 10 min. Second, acifluorfen was added (500 μM) followed by 15 min incubation to displace noncovalently bound ligand. Specific covalent binding was considered to be the difference between total and nonspecific covalent binding, i.e., with 0 and 10 μM THP, respectively. [b] Normalized to 100%. [c] Apparent covalent derivatization equivalent to 13% of the added protox.

in the presence or absence of substrate (5 μM), establishing that DPI and [^{3}H]AzTHP do not share overlapping binding sites. Binding assays containing protoporphyrinogen show the red color of oxidized substrate inversely related to DPI concentration as expected for enzymatically active incubations. Substrate inhibition of [^{3}H]AzTHP binding was not examined because enzyme oxidation of protoporphyrinogen reduces its levels to below saturation concentrations after 2 min.

DPI irreversibly inhibits protox activity in yeast mitochondrial membranes at high potency (K_i = 67.5 nM) with maximal inhibition observed after 5 min (*18*). Unfortunately, a direct comparison between DPI inhibition results with human protox (this study) and yeast (*18*) is not possible because of differences in the two assay systems. Protoporphyrinogen was consumed too rapidly in assays with solubilized human protox to maintain saturation for longer than 2 min, whereas with membranous yeast mitochondrial protox, assays were run for 20 min or more. Also, this study involved no reducing agents and the enzyme was detergent solubilized, whereas the yeast protox assays used buffer containing dithiothreitol and membranous enzyme. The effects of these factors on DPI protox inhibitory potency are unknown.

Photoaffinity Labeling of Human Protox with [^{3}H]AzTHP. The first step has been taken in photoaffinity labeling by incubating protox with [^{3}H]AzTHP for 15 min in the dark (to achieve near saturation of the specific binding site), then exposure to 300 nm light for 10 min which completely photodecomposes the radioligand and yields photoproducts which do not interfere with the binding. Without light, there is no covalent binding and the specific binding is completely protected by unlabeled ligand (10 μM THP). Following irradiation, 26% of observed covalent binding (nondisplaceable by 500 μM acifluorfen) is specific as determined by the difference of total and nonspecific binding (Table 1). When the specifically bound radioligand is calculated on a molar equivalent basis, with correction for loss on filtration, the apparent covalent labeling is 13% corresponding to 6.9 μg of the 53 μg protox used. This extent of covalent labeling is not in itself a limitation because of the high specific activity of [^{3}H]AzTHP and the potential availability of large quantities of pure protox.

Conclusions. N-Aryl-tetrahydrophthalimides have three advantages as candidate photoaffinity probes: the 4-chloro-

2-fluoro analogues have outstanding potency and allow placement of a photoreactive moiety at the 5-position (this study); halogens ortho to the azido substituent are expected to improve photoreactivity and reduce the possibility of the excited nitrene undergoing ring expansion (*35*); the tetrahydrophthalimide moiety is easily radiolabeled at high specific activity (*8*). There are also two disadvantages of the *N*-aryltetrahydrophthalimides, that is, their instability in basic conditions and reactivity with thiols (*8*, *13*). Standard approaches for analyzing polypeptides to assign the position of photoaffinity labeling involve digestion with proteases and electrophoresis both under alkaline conditions and the latter frequently with thiols. Further studies on the structure of the protox active site photoaffinity labeled with [^{3}H]AzTHP will be most appropriate under acidic conditions (that is, cyanogen bromide degradation and acetic acid-urea electrophoresis) to minimize hydrolysis which would cleave the tetrahydrophthalimide label from the azidophenyl-derivatized peptide.

Protox contains one herbicide binding site per FAD (this study) and one FAD per homodimer (*3*) indicating one herbicide/substrate binding site per homodimer. The enzyme activity is inhibited by DPI on derivatizing FAD without affecting herbicide binding, thereby establishing that the FAD site may not be proximal to the herbicide/substrate site. These binding site relationships will be further clarified by defining the peptide regions undergoing photoaffinity labeling with radioiodinated DPI (*18*) and [^{3}H]AzTHP.

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SUPPORTING INFORMATION AVAILABLE

Full characterization data on compounds **1**−**4** and AzTHP (2 pages). Ordering information is given on any current masthead page.

REFERENCES

1. Camadro, J.-M., Thome, F., Brouillet, N., and Labbe, P. (1994) *J. Biol. Chem. 269*, 32085−32091.
2. Dailey, T. A., Dailey, H. A., Meissner, P., and Prasad, A. R. K. (1995) *Arch. Biochem. Biophys. 324*, 379−384.
3. Dailey, T. A., and Dailey, H. A. (1996) *Protein Sci. 5*, 98−105.
4. Corrigall, A. V., Hift, R. J., Adams, P. A., and Kirsch, R. E. (1994) *Biochem. Mol. Biol. Int. 34*, 1283−1289.
5. Duke, S. O., and Rebeiz, C. A. (Eds.) (1994) *Porphyric pesticides: chemistry, toxicology, and pharmaceutical applications*, ACS Symposium Series 559, 317 pp, American Chemical Society, Washington, DC.
6. Fingar, V. H., Wieman, T. J., McMahon, K. S., Haydon, P. S., Halling, B. P., Yuhas, D. A., and Winkelman, J. W. (1997) *Cancer Res. 57*, 4551−4556.
7. Deybach, J. Ch., de Verneuil, H., and Nordmann, Y. (1981) *Hum. Genet. 58*, 425−428.
8. Birchfield, N. B., and Casida, J. E. (1996) *Chem. Res. Toxicol. 9*, 1135−1139.
9. Nicolaus, B., Johansen, J. N., and Böger, P. (1995) *Pestic. Biochem. Physiol. 51*, 20−29.
10. Matringe, M., Mornet, R., and Scalla, R. (1992) *Eur. J. Biochem. 209*, 861−868.
11. Camadro, J.-M., Matringe, M., Scalla, R., and Labbe, P. (1991) *Biochem. J. 277*, 17−21.
12. Matringe, M., Camadro, J.-M., Labbe, P., and Scalla, R. (1989) *FEBS Lett. 215*, 38.
13. Birchfield, N. B., and Casida, J. E. (1997) *Pestic. Biochem. Physiol. 57*, 36−43.
14. Lee, H. J., Duke, M. V., and Duke, S. O. (1993) *Plant Physiol. 102*, 881−889.
15. Retzlaff, K., and Böger, P. (1996) *Pestic. Biochem. Physiol. 54*, 105−114.
16. Devine, M., Duke, S. O., and Fedtke, C. (1993) in *Physiology of Herbicide Action*, pp 177−188, Prentice Hall, Englewood Cliffs, NJ.
17. Dailey, H. A. (Ed.) (1990) *Biosynthesis of Heme and Chlorophylls*, McGraw-Hill, New York.
18. Arnould, S., Berthon, J.-L., Hubert, C., Dias, M., Cibert, C., Mornet, R., and Camadro, J.-M. (1997) *Biochemistry 36*, 10178−10184.
19. Wang, J. M., Asami, T., Che, F. S., Murofushi, N., and Yoshida, S. (1995) *Ann. Meeting of Pesticide Science Society of Japan, Abstract*, Tokyo, Japan, p 82 (in Japanese).
20. O'Connor, N., Mornet, R., Matringe, M., Clair, D., Scalla, R., Fujimoto, T. T., and Swithenbank, C. (1994) *Bioorg. Med. Chem. 2*, 339−342.
21. Camadro, J.-M., Matringe, M., Thome, F., Brouillet, N., Mornet, R., and Labbe, P. (1995) *Eur. J. Biochem. 229*, 669−674.
22. Dailey, T. A., personal communication.
23. Dailey, T. A., and Dailey, H. A. (1997) *Methods Enzymol. 281*, 340−349.
24. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
25. Camadro, J.-M., Matringe, M., Scalla, R., and Labbe, P. (1993) in *Target assays for modern herbicides and related phytotoxic compounds* (Boger, P., and Sandmann, G., Eds.) pp 29−34, CRC Press/Lewis Publishers, Boca Raton, FL.
26. Brenner, D. A., and Bloomer, J. R. (1980) *Clin. Chim. Acta 100*, 259−266.
27. Goddard, S. J. (1977) U.S. Patent 4032326.
28. Nagano, E., Hashimoto, S., Yoshida, R., Matsumoto, H., and Kamoshita, K. (1984) U.S. Patent 4484940.
29. Latli, B., Morimoto, H., Williams, P. G., and Casida, J. E. (1998) *J. Labelled Compd. Radiopharm. 41*, 191−199.
30. Bellamy, F. D., and Ou, K. (1984) *Tetrahedron Lett. 25*, 839−842.
31. Gee, K. R., Durant, G. J., Holmes, D. L., Magar, S. S., Weber, E., Wong, S. T., and Keana, J. F. W. (1993) *Bioconjugate Chem. 4*, 226−229.
32. Nandihalli, U. B., Duke, M. V., and Duke, S. O. (1992) *Pestic. Biochem. Physiol. 43*, 193−211.
33. Lopez, J. M., Imperial, S., Valderrama, R., and Navarro, S. (1993) *Clin. Chim. Acta* (Netherlands), *22*, 91−100.
34. O'Donnell, V. B., Smith, G. C. M., and Jones, O. T. G. (1994) *Mol. Pharmacol. 46*, 778−785.
35. Karney, W. L., and Borden, W. T. (1997) *J. Am. Chem. Soc. 119*, 3347−3350.

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