Evaluation of 3-Azidiamantane as Photoaffinity Probe of Cytochrome P450

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Abstract. 3-azidiamantane (DIA-N_2) has been shown to be a photolabile carbene-generating probe interacting specifically with cytochrome P450 (P450) active centre. To evaluate the modification of P450 by the probe, radiolabelled [9-^{3}H]-3-azidiamantane was prepared by reductive dehalogenation of its precursor, 3-oxo-9-bromodiamantane ethylene ketal. The synthesis was optimized as the proper precursor and reaction conditions were concerned to produce 96% pure product (overall yield 59%). An incorporation efficacy of the probe photoactivated at 366 nm was examined with two different proteins, BSA and rat phenobarbital-inducible P450 2B1, both having hydrophobic binding sites. Under photolysis the photoaffinity probe generated short-lived (> 90%) intermediates binding immediately to the protein. The yield of photoactivated DIA-N_2 incorporation was 12% and 11% for BSA and P450, respectively. The presence of reduced glutathione, a scavenger of reactive intermediates, did not affect the probe incorporation markedly. On the other hand, scavengers entering the P450 active centre, methanol and dithiothreitol, reduced the protein labelling by 36% and 42%, respectively. Similarly, at DIA-N_2, aminopyrine (substrates), and metyrapone (inhibitor) 50 times molar excess over the probe, prevented its binding by about 40%. In addition, when photoaffinity labelling was carried out with microsomal preparation, the substrate with a high affinity for the P450 2B1, diamantane, (at 20 times molar excess to the probe) caused 47% inhibition of the P450 covalent labelling. These results, suggesting a high specificity of the probe binding, show that it can be applied as a photoaffinity probe for cytochrome P450 2B1 active centre studies.

Key words: Cytochrome P-450 — Photoaffinity labelling — Photolysis — 3-Azidiamantane

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Introduction

Cytochromes P450 (P450s) are heme-containing enzymes which have been in the focus of research interests for several decades because of their involvement in the metabolism of foreign compounds, namely drugs and carcinogens (Guengerich 1988). In addition, these enzymes play an important role in the biosynthesis of essential endogenic compounds like fatty acids, steroids, prostaglandins and vitamins D (McGiff 1991, Okuda 1994, Salaun and Helvig 1995). In eucaryotic organisms, P450s are integral membrane proteins of the endoplasmic reticulum, mitochondria, and the nucleus of various tissue cells. The membrane location makes investigation of their structure and function difficult since no membrane cytochrome P450 has been crystallized and examined with X-ray crystallography as yet. On the other hand, four microbial P450s (cytosolic proteins) were successfully crystallized and their 3D-structure revealed (Poulos et al. 1987, Ravichandran et al. 1993, Hasemann et al. 1994, Cupp-Vickery and Poulos 1995). Although there is some degree of sequence homology and expected domain-structure similarities between eucaryotic and prokaryotic P450s the adoption of bacterial P450 structures as a model to understand e.g. mammal P450 active centre structures is rather limited (Sŏklarz and Halpert 1997).

Among the various methods to study cytochrome P450 active centre, site-directed mutagenesis of important amino acid residues (Strobel and Halpert 1997), covalent labelling using reactive derivatives of specific ligands (Roberts et al. 1994), and spectroscopic approaches (Myers et al. 1994), are employed most frequently. Photoaffinity labelling is one of the promising covalent labelling techniques applicable to the labelling of amino acid residues in the enzyme active centre (Chowdhry and Westheimer 1979, Fleming 1995). This technique, developed on the principles of affinity labelling, makes an advantage of "in situ" photolytically generated extremely reactive intermediates of a photolabile ligand bound in the enzyme active centre. In selecting a photoaffinity probe, the most important criterion is the probe binding affinity for the enzyme as well as the reactivity of photolytically produced intermediates; the latter is essential especially for the labelling of P450 hydrophobic active centres. Both requirements are met with 3-azidamantane, a carbene generating photoaffinity probe derived from the hydrocarbon diamantane. It is well documented that diamantane is a highly selective substrate of cytochrome P450 2B1 (Hodek et al. 1988). This compound and its derivatives were used to study the P450 2B1 active site (Hodek et al. 1995). Moreover, photolysis of 3-azidiamantane (diamantane photolabile derivative) yields diamantane carbene (see Figure 1) which shows an excellent ability to bind to non-activated hydrocarbons (e.g. hexane) similar to the side chains of the hydrophobic amino acid residues assumed to be present in the cytochrome P450 active centre (Hodek et al. 1997). Recently, two photoaffinity probes, diazidodesmethylbenzphetamine and azidoc-

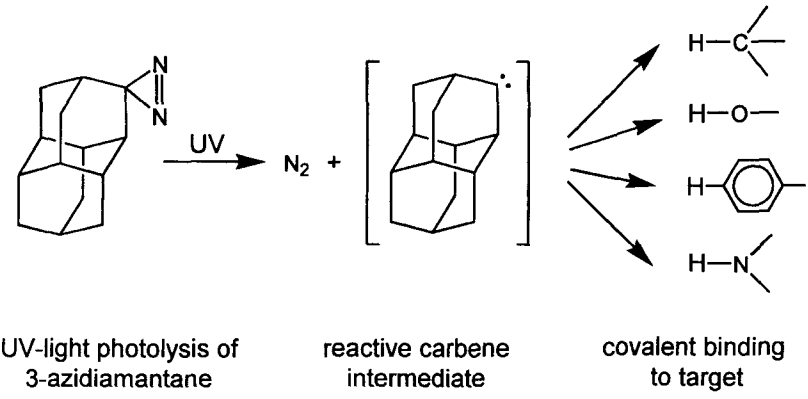

Figure 1. Scheme of 3-azidiamantene photolysis resulting in carbene reacting with various amino acid residues of a target protein

umene, were successfully used to identify peptides within different P450 substrate binding sites (Cvrk et al. 1996, Cvrk and Strobel 1998).

The present paper describes the preparation of a radiolabelled derivative of 3-azidiamantane and the examination of its binding (after photoactivation) to cytochrome P450 2B1 and to a model protein, BSA.

Abbreviations: BSA, bovine serum albumin; DIA, diamantane (pentacyclo/7,3,$1,1^{4,12},0^{2,7},0^{6,12}$/tetradecane); DIA-$N_2$, 3-azidiamantane; DLPC, L-α-dilauroyl phosphatidylcholine; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate; GSH, reduced glutathione; [^{3}H]DIA-N_2, tritium-labelled derivative of 3-azidiamantane: MeOH, methanol; P450, cytochrome P450; PB-Ms, phenobarbital-induced microsomes; SDS, sodium dodecylsulphate; vit. E, O-acetyl-α-tocopherol;

Materials and Methods

Materials

PB-induced microsomes and cytochrome P450 2B1 (specific content 14.1 nmol/mg) were prepared from phenobarbital treated male rats (Wistar, 110-130 g) as described previously (Anzenbacher et al. 1984, Hodek et al. 1988). Diamantane (pentacyclo/7,3,$1,1^{4,12},0^{2,7},0^{6,12}$/ tetradecane), 3-oxodiamantane, 5-chloro-3-oxodiamatane, and 3-oxo-9-hydroxydimantane were gifts from Ing. Janků (Prague Institute of Technology, Czech Republic). 3-Azidiamantane, spiro-(diazirine-3,3′-diamantane), was synthesised by the optimized procedure of Vodička et al. (1985). Hydroxyl-

amine-O-sulfonic acid was prepared according to Schmitz et al. (1967) and its purity was determined iodometrically. Phenobarbital sodium salt was purchased from Farmakon (Olomouc, Czech Republic). Bovine serum albumin was a product of Imuna (Šarišké Michalany, Slovakia). NADPH, dithiothreitol, L-α-dilauroyl phosphatidylcholine and reduced glutathione were obtained from Fluka (Switzerland). O-acetyl-α-tocopherol was purchased from Léčiva (Prague, Czech republic). All other chemicals used were of analytical grade or better.

Analytical methods

Protein concentration in samples was determined by the Lowry (1951) or the Bradford (1976) method with bovine serum albumin as a standard. P-450 content was measured based on a reduced cytochrome P-450 complex with CO (Omura and Sato 1964). SDS-gel electrophoresis was performed with 7.5% gel in a discontinuous system according to Laemmli (1970).

All the diamantane derivatives and intermediates prepared when optimizing the synthesis of radiolabelled 3-azidiamantane were analysed and identified by means of mass spectroscopy (Table 1) on Finnigan MAT (USA) connected with GC Varian 3400 (DB-5 column, 30 m). The radioactivity of the samples was determined on a liquid scintillation counter Isocap/300 (Nuclear, Chicago, USA).

Table 1. Mass spectroscopy data of diamantane derivatives

Compound	M^+	Other fragments
I	218	201, 200, 172, 145, 130, 117, 105, 91
II	280	201, 172, 145, 131, 117, 105, 91, 80
III	324	245, 203, 173, 131, 117, 105, 91, 81, 79
IV	246	204, 174, 131, 117, 105, 91, 79
V	202	174, 145, 130, 117, 105, 91, 79
VI	*	186, 171, 157, 143, 129, 117, 91, 79
VII	*	186, 171, 157, 143, 129, 117, 91, 79

* Molecular ion (M^+) not detected – spectrum after elimination of the nitrogen molecule

Photoaffinity probe synthesis

First, synthesis of 3-hydrazidiamantane (Vodička et al. 1985) was optimized in respect to the molar ratio of reactants to result in a high yield of the product. In a stirred Reacti-Vial (Pierce, USA) under cooling (5 °C) 5 mg of 3-oxodiamantane was dissolved in dry methanol (0.2 ml) containing 10–35% ammonia, and methanol solution of hydroxylamine-O-sulfonic acid (2–5 mg in 0.1 ml) was added through

a septum. After stirring for 1 h at 10 °C the reaction was allowed to proceed for 2 days at 5 °C. The solvent was evaporated from the reaction mixture and the residue extracted with chloroform. Products of different reaction runs were compared on TLC developed with chloroform-acetone (3:2). 3-Hydrazidiamantane was visualized by spraying the plate with acidified ethanol solution of potassium iodide.

Then, oxidation of 3-hydrazidiamantane to 3-azidiamantane by chromium(VI) oxide (Vodička et al. 1985) and silver oxide (Schmitz 1964) was tested. To the reaction mixture containing 1.5 mg of 3-hydrazidiamantane dissolved in acetone (150 μl) and 2 mol/l sulfuric acid (20 μl), 15 μl of 0.9 mmol/l chromium(VI) oxide (in 2 mol/l sulfuric acid) was added under stirring. After 15 min. the reaction mixture was diluted with water (4 ml) and then extracted to tert. butyl methyl ether (4 × 0.5 ml). Alternative oxidation was performed with silver oxide (80 mg) stirred at 5 °C overnight in a solution of 3-hydrazidiamantane (1.5 mg) in diethyl ether. The reaction mixture was extracted to chloroform. Products of both reactions were examined by TLC developed with hexane.

[^{3}H]DIA-N_2 was prepared using a modified approach originally described by Bayley and Knowles (1980) for 3-aziadamantane. The scheme of the synthesis is shown in Fig. 2. Diamantane derivatives separated on TLC plates (Silufol, Kavalier, Czech Republic) were detected as fluorescent spots after spraying the plate with eosine in ethanol.

Briefly, 9-bromo-3-oxodiamantane [**II**] was prepared from 9-hydroxy-3-oxodiamantane [**I**] (100 mg) by overnight reaction with phosphorus tribromide (100 μl) in dry benzene at 60 °C yielding 95% product. The oxo-group of the product was converted to ethylene ketal [**III**] by overnight boiling **II** (50 mg) in acidified benzene (15 ml) with addition of ethylene glycol (1 ml). Product **III** was separated as a benzene solution after the reaction mixture neutralization by 5% sodium carbonate. Reductive dehalogenation by exposure of **III** to tritium gas on a Pd/C catalyst resulting in tritiated diamantane ethylene ketal [**IV**] was performed by UVVVR (Prague, Czech Republic). The ethylene ketal protecting group was cleaved by 12 h stirring of a mixture consisting of **IV** (about 1 mg) dissolved in dioxane (0.3 ml), conc. sulfuric acid (10 μl) and potassium periodate (10 mg) added to 0.2 ml of water. To reduce the specific radioactivity of the released 3-oxodiamantane [**V**], 4 mg of the non-labelled compound were added as a carrier. The reaction mixture was neutralized by sodium carbonate and the product was extracted with hexane. 2.5 mCi (about 2.5 mg) of **V** dissolved in 25% ammonia solution in methanol (0.1 ml) was reacted with hydroxylamine-O-sulfonic acid (1.7 mg) injected as a methanol solution (55 μl) through a septum to the reaction mixture. While stirring, the reaction was allowed to proceed for 3 h at 0 °C and then for additional 5 days at 5 °C. The solvent was evaporated from the reaction mixture and the residue extracted with chloroform. The product, 3-hydrazidiamantane [**VI**] dissolved in diethyl ether (0.4 ml) was oxidized by silver oxide (60 mg) under stirring at room

temperature for 3 h. The resulting 3-azidiamantane [**VII**] was extracted from the reaction suspension with tert. butyl methyl ether (5 × 1 ml). Crude **VII** was purified on a silica gel column (0.6 × 10 cm) using hexane as the mobile phase. The concentration of pure **VII** in hexane was measured spectrophotometrically using $\varepsilon_{375-450} = 172\ cm^{-1}.\ mol^{-1}.\ l$ determined for non-radioactive 3-azidiamantane.

Introductory radiolabelling experiments were carried out with the other probe precursors: 3-oxodiamantane, 5-chloro-3-oxodiamantane, 5-chloro-3-oxodiamantane ethylene ketal, and 9-bromo-3-oxodiamantane using lithium tritide or tritium (g) as a source of tritium to be incorporated into the precursor molecule. To restore potentially reduced oxo-group, labelled compounds (1–2 mg) dissolved in pyridine (150 μl) were oxidized, under stirring, by chromium(III) oxide (40 μl, 4 mg/ml) for 16 h at room temperature. The whole reaction mixture was applied to a silica gel column (1 × 8 cm) and the products were eluted with hexane-diethyl ether (1:2). Pure 3-oxodiamantane (and/or ethylene ketal) were converted to radiolabelled probe as described above.

Photolytic experiments

Photolyses were carried out using a photolyser equipped with a medium pressure mercury arc lamp RVK 125 (Tesla, Prague, Czech Republic) emitting largely UV light of 366 nm, and water cooled (5 °C) holder for Pyrex tubes (4 mm i.d.) positioned at a distance of 2.5 cm from the UV-light source. To characterize the probe labelling efficacy and specificity, photolytic experiments were performed with radiolabelled [^{3}H]DIA-N_2 (diluted with non-radiolabelled compound) in solutions of BSA, cytochrome P450 2B1 or PB-induced microsomal preparations. The protein samples (0.55 ml) diluted to 1 mg/ml by 100 mmol/l potassium phosphate buffer pH 7.4 containing 0.1 mmol/l EDTA were photolyzed for 15 min in the presence of 14 μmol/l [^{3}H]DIA-N_2 and one of the following perturbants: 50 mmol/l (or 13 mmol/l) GSH, 50 mmol/l DTT, 0.7 mmol/l DIA-N_2, 0.7 mmol/l aminopyrine, 0.7 mmol/l metyrapone, 900 mmol/l MeOH, 500 mmol/l DMSO. Diamantane derivatives were added from stock (MeOH or DMSO) solutions.

For photolytic experiments in the absence of organic solvents DLPC suspensions of [^{3}H]DIA-N_2, DIA or vit. E were used to introduce the compounds to the protein solutions. The final concentrations of DLPC, DIA and vit. E were adjusted to 20 molar excess over the probe [^{3}H]DIA-N_2 (9 μmol/l) in the reaction mixture. In the case of microsomal preparations the final concentration of DLPC was by 5 times increased.

To detect long-lived diamantane derivatives generated by photolysis of the probe BSA (1 mg/ml) was added to the reaction mixture containing [^{3}H]DIA-N_2 (14 μmol/l) either prior or after the probe photolysis within 1 min or 48 hours. Photolyzed samples as well as controls (non-photolyzed samples) were subjected to organic solvent extraction to remove non-covalently bound diamantane derivatives.

In order to minimize the retention of extractable radioactivity, 10 μl of the solution containing 20 mmol/l DIA-N_2 and 6 mmol/l 3-hydroxydiamantane were added to the reaction mixture (550 μl). Then, the reaction mixture was 3 times extracted with 4 ml of tert. butyl methyl ether + chloroform, 2:5 followed by 5 min centrifugation at 800 × g to separate water and organic solvents layers. In the final step, 4 ml of acetone and 20 μl of conc. sulfuric acid was added to the water layer and left to precipitate for 10 min. The precipitate was sedimented by centrifugation (see above) and washed with a new portion of acetone.

Air-dried sediments dissolved in 0.5 ml of 10 mol/l urea and 0.5 ml of 2 mol/l sodium hydroxide were used to determine their protein content. The other sample aliquot was neutralized by acetic acid and its radioactivity measured after mixing with Insta Gel (Packard, USA) scintillation solution. Microsomal samples were prepared for SDS-electrophoresis by resuspending the sediment in reducing sample buffer (400 μl) only, sonication and neutralization by ammonia carbonate prior to the application on the gel (400 μg/well). After microsomal protein separation tracks of the gel were cut into 4 mm slices, solubilized by 16 h incubation at 70 °C with a mixture of 30% ammonia hydroxide and 30% hydrogen peroxide (5:95). The radioactivity of the solubilized slices was determined by scintillation counting.

In pilot studies, the non-covalently bound radioactivity was either extracted by a mixture of tert. butyl methyl ether-chloroform or acetone or by micro-scale chromatography of Sephadex LH-20 (1.5 ml) followed by acetone precipitation.

Results and Discussion

Synthesis of [9-^{3}H]-3-azidiamantane

The radiolabelled photoaffinity probe of a high specific radioactivity (146 Ci/mol) was prepared applying a multi-step synthesis outlined in Figure 2. The radiochemical yield (after two last reaction steps and purification) and the purity of the final [^{3}H]DIA-N_2 were 59% and 96%, respectively. In methanol solution [^{3}H]DIA-N_2 was stable, showing > 90% radiochemical purity after storage for one year at 5 °C. It is a great advantage of 3-azidiamantane over the other diazirine probes, e.g. 3-(trifluormethyl)-3-(iodophenyl)diazirine (Brunner and Semenza 1981), which degrade 3% every two weeks.

To select the proper diamantane derivative for tritium introduction at the beginning of the probe preparation, the following derivatives were tested using different tritium sources: 3-oxodiamantane, 5-chloro-3-oxodiamantane, 5-chloro-3-oxodiamantane ethylene ketal, 9-hydroxy-3-oxodiamantane, and 9-hydroxy-3-oxodiamantane ethylene ketal. The results of their exposure to tritium (g) or lithium tritide are summarized in Table 2. It is obvious that the best results in respect to incorporated radioactivity and product purity were provided by reductive de-

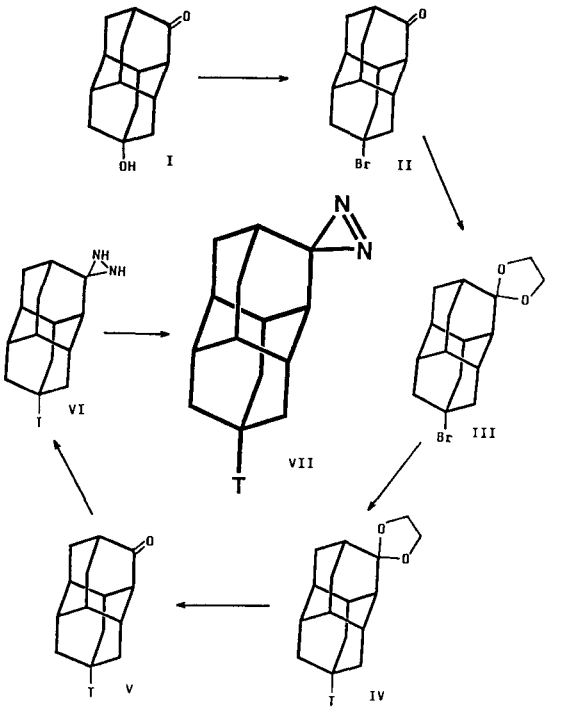

Figure 2. Scheme of tritium (T) labelled 3-azidiamantane synthesis Radiolabelled dia mantane derivatives are numbered as follows 9-hydroxy-3 oxodiamantane, **I**, 9 bromo-3 oxodiamantane, **II**, 9-bromo-3-oxodiamantane ethylene ketal, **III**, 3-oxodiamantane ethylene ketal, **IV** 3 oxodiamantane, **V**, 3 hydrazidiamantane **VI**, 3-azidiamantane, **VII.**

halogenation using tritium gas It remains unclear, why lithium tritide failed in the labelling reaction Comparing the precursors tested, the bromo derivative was proved to be a better choice for tritium introduction than the chloro-derivative which was > 10 times less reactive Since in the course of the bromine atom replacement with tritium also oxo-group gets reduced, the protection of the oxo-group as ethylene ketal is advantageous

The conversion of radiolabelled 3-oxodiamantane [**V**] to 3-hydrazidiamantane [**VI**] was optimized with respect to the amounts of ammonia and hydroxylamine-O-sulfonic acid necessary for the reaction to be accomplished The optimal concentra tions established were 80 mmol/l for **V**, 100 mmol/l for hydroxylamine-O-sulfonic acid, and 10 mol/l for ammonia in methanol Any change of the reactant concentrations resulted in a significant drop in the reaction yield (data not shown)

In contrast to reports by others (Bayley and Knowles 1980, Vodička et al

Table 2. Preparation of radiolabelled photoaffinity probe precursor

Source compound	Tritium source	Incorporated radioactivity^{+} [Ci/mmol]	Identified radioactive diamantane derivative* (%)
3-oxodiamantane	T_2	0 08	3-hydroxy (51) 3-oxo (14)
5-chloro 3-oxodiamantane	T_2	0 06	3-hydroxy (35) 3-oxo (8)
9-bromo-3-oxodiamantane	T_2	0 75	3-hydroxy (13) 3-oxo (22)
5-chloro-3-oxodiamantane ethylene ketal	LiT	0 09	3-hydroxy (49) 3 oxo (10) 3-oxo-ethylene ketal (2)
9-bromo-3 oxodiamantane ethylene ketal	LiT	0 05	3-hydroxy (38) 3-oxo (18)
9 bromo-3-oxodiamantane ethylene ketal	T_2	3 46	3-hydroxy (2) 3 oxo (5) 3-oxo-ethylene ketal (63)

T_2 tritium (g), LiT lithium tritide, $^{+}$ radioactivity incorporated per 1 mmol of source compound, * percentage of total radioactivity co-migrating with the corresponding diamantane derivative standard

1985) oxidation of **VI** to **VII** by chromium(VI) oxide resulted in non-satisfactory reaction yields (about 55%) as well as a low product purity (62%) That is why oxidation by the silver oxide procedure was used to obtain product **VII** of 95% radiochemical purity and 78% yield

Using the indicated molar ratio of reactants for the synthesis of **VI** in conjunction with silver oxide oxidation of **VI,** even micro-scale reaction (volume 155 μl) yielded higher amounts of **VII** than those obtained in a regular scale by Vodička et al (1985)

Photolytic experiments

In order to evaluate the photoaffinity probe, [^{3}H]DIA-N_2, from the aspects of the labelling yield and specificity, photolysis of the probe was carried out with BSA, cytochrome P450 2B1 or PB-induced microsomal preparations

First, the technique enabling to remove a great majority of non-covalently bound radiolabelled diamantane derivatives after photolysis was developed Only the procedure combining extraction of the reaction mixture with water non-miscible solvents and acetone precipitation in the presence of protein collector (see Materials and Methods) resulted in high yields of protein recovery (> 70%) and a residual radioactivity close to the background value The separation efficacy was repro-

ducibly 99.97% based on the original radioactivity added. Other techniques tested were less effective in respect to the removal of diamantane derivatives. E.g., the use of Sephadex LH 20 resulted in the retention of 6.7% the original radioactivity in the purified sample.

Since the occurrence of long-lived intermediates of the photoaffinity probe, produced as a rearrangement and/or direct photolysis products, is one of the factors limiting the labelling specificity of photoaffinity probes and thus their proper application, it is of a great importance to examine the presence of these intermediates. For this purpose, time course experiments with BSA as a model protein with hydrophobic binding sites were carried out. BSA was present during the photolysis of [^{3}H]DIA-N_2, or was added either immediately after the probe photolysis or much later (48 h) when the long-lived intermediates were presumed to be inactive. The data in Table 3 show that the probe was preferentially photolyzed into short-lived species labelling fast the protein binding site. Only a very small fraction of the probe was converted upon photolysis into a long-lived derivative, most likely 3-diazodiamantane, which accounted for the BSA labelling after its irradiation. These results are in accordance with those obtained with the photolysis of non-radioactive 3-azidiamantane in model systems (Hodek et al. 1997).

Table 3 Incorporation of photoactivated 3-azidiamantane to BSA

Presence of BSA	Incorporated radioactivity [DPM]	Recovered protein [μg]	Relative labelling [DPM/mg]
during photolysis	4384 ± 503	264 ± 7	16630 ± 1730
1 min after photolysis	394 ± 49	275 ± 9	1430 ± 120
48 h after photolysis	97 ± 3	336 ± 19	290 ± 10

The photolysis was carried out using 1 mg/ml BSA solution and 14 μmol/l [^{3}H]DIA-N_2 (87818 DPM/ml) The data are means of three parallel experiments ± standard errors

As the photoaffinity probe is almost insoluble in water, organic solvents like methanol or dimethyl sulfoxide had to be used to introduce the probe into the reaction mixture. Methanol (MeOH) was found (Hodek et al. 1997) to be a powerful acceptor of photoactivated probe. In addition, dimethyl sulfoxide (DMSO) is a well known radical scavenger. Thus the presence of these compounds during the photolysis experiments was expected to affect the extent of the probe labelling Hence, photolysis in the presence of the tested solvents (cca 0.2% in the final dilution) was carried out with BSA and P450 samples. Data in Fig. 3 show that

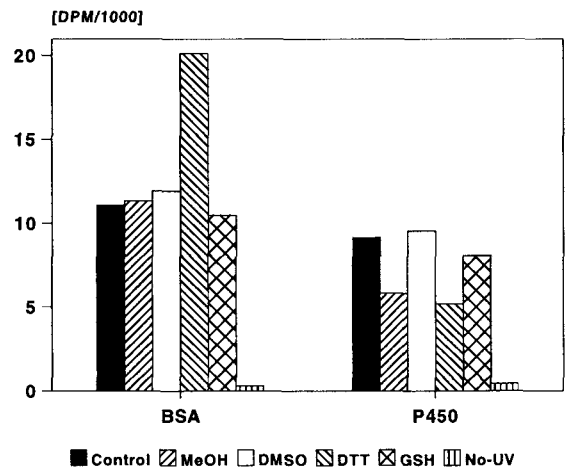

Figure 3. Effect of solvents, methanol (**MeOH**) or dimethyl sulfoxide (**DMSO**), and scavengers, dithiothreitol (**DTT**) or reduced glutathione (**GSH**), on BSA or cytochrome P450 2B1 labelling The protein samples (1 mg/ml) in 100 mmol/l potassium phosphate buffer pH 7 4 containing 0 1 mmol/l EDTA and 14 μmol/l [^{3}H]DIA-N_2 were photolyzed for 15 min in the absence (**Control**) or presence of either 900 mmol/l MeOH or 500 mmol/l DMSO or 50 mmol/l DTT or 50 mmol/l GSH Sample, **No-UV**, was not irradiated Data are means of three parallel experiments (the standard errors did not exceed 10% for MeOH, DMSO and 5% for DTT, GSH)

there was no considerable effect of the used solvent on the BSA labelling, indicating that none of these compounds are able to enter the BSA hydrophobic binding sites However, in the case of experiments with P450 and the probe photolyzed in the presence of MeOH or DMSO (see Fig 3) the extent of the labelling differed markedly While DMSO had no or negligible stimulatory effect, MeOH caused 36% inhibition of the P450 labelling This result can be explained by the ability of these solvents to bind to the cytochrome P450 active site methanol, like the other alcohols, is a potent heme iron ligand of cytochrome P450 (Backes and Canady 1981) Because of its reactivity MeOH is able to quench the photoactivated probe more readily than do water molecules which are assumed to also be present in the active site On the other hand, DMSO, displaying a rather low binding affinity for cytochrome P450, did not affect the labelling An increased extent of both BSA and P450 labelling in the presence of DMSO might originate from DMSO ability to serve as a better solvent of the probe than MeOH, assuring a higher probe concentration in the reaction mixture than does MeOH

DTT and GSH are recommended to be used in order to increase the probe

labelling specificity (Bayley 1983) These scavengers are able to react with photoac tivated probe outside the binding site and thus reduce non-specific labelling of the target macromolecule The results of experiments examining these compounds for BSA and P450 2B1 photoaffinity labelling are also summarized in Fig 3 GSH did not reduce labelling markedly Surprisingly, DTT almost doubled the yield of the probe incorporation to BSA The reason underlying this phenomenon is unclear It might be explained by the reduction of BSA S-S bond by one of the DTT SH group, while the other SH-group of the DTT molecule is modified with the probe On the other hand, with P450 system DTT reduces the probe labelling by 43% DTT, like MeOH, is able to bind to the P450 2B1 active site (Dawson and Masanori 1987) and thus to quench the probe present there GSH, a compound which hardly binds to the P450 active site, induced an only 12% decrease in the labelling yield As suming that all of the probe gets quenched in the solution surrounding the protein molecule, the data strongly suggest a highly specific incorporation of the activated probe

As specific labelling is considered to be saturable in contrast to the non specific one, competition experiments using excess of compounds binding to the active site were carried out All these compounds, substrates (aminopyrine and 3-azidiamantane (added in DMSO)), and the P450 2B1 selective inhibitor metyra pone, in 50 times molar excess were able to reduce the labelling by the photoacti vated probe to a similar extent, approximately 40% (see Fig 4) Moreover, when 3 azidiamantane was added in methanol solution the effects of both perturbants combined the probe binding decreased by 65% This result, reflecting the additive effect of both compounds, revealed that a small ligand like MeOH and a bulky substrate e g 3-azidiamantane are able to bind to P450 2B1 simultaneously

Subsequent experiments were done to examine the ability of vitamin E (an- other radical scavenger) and diamantane, as one of the best substrate of P450 2B1 (Hodek et al 1988) to affect the probe binding The results are illustrated in Fig 5 As both compounds are hardly soluble in polar solvents, DLPC suspension was used to introduce them into the reaction mixture In other words, solvents were substituted with phospholipid to carry the compounds Vitamin E was found not to enter the P450 active site (not published results) Thus, one could expect a similar decrease of the radiolabelled probe incorporation like with GSH Indeed, the probe binding was decreased by only 12% In addition, diamantane showing about four times higher binding affinity for the P450 2B1 active site than 3-azidiamantane (Hodek et al 1997), was the most efficient competitor tested In the presence of diamantane the covalent labelling of cytochrome P450 was prevented to 46% even when only a 20-fold molar excess of competitor (DIA) over the probe was used When both compounds (DIA + vit E) were combined in the reaction mixture, the labelling yield decreased to 39% of the unaffected control experiment Again, some additive effect of combined compounds was observed

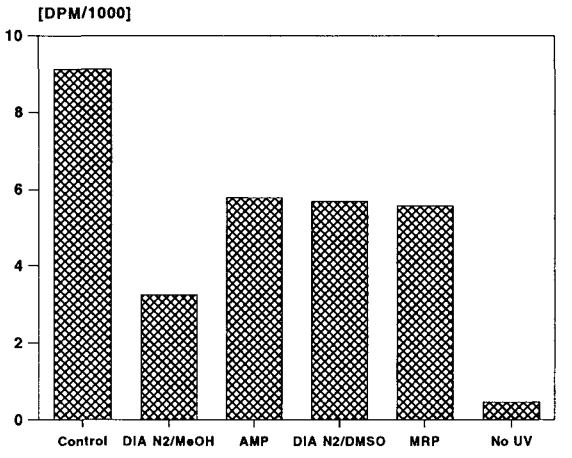

Figure 4. Photoaffinity labelling of cytochrome P450 2B1 with 14 μmol/l [^{3}H]DIA-N_2 in the presence of 0 7 mmol/l competitors **Control**, no competitor, **DIA-N2/MeOH**, 3-azidiamantane (in methanol), **AMP**, aminopyrine, **DIA-N2/DMSO,** 3-azidiamantane (in dimethyl sulfoxide), **MRP**, metyrapone, **No-UV**, no photolysis Reaction mixtures containing 1 mg of P450/ml of 100 mmol/l potassium phosphate buffer pH 7 4, 0 1 mmol/l EDTA were photolyzed for 15 min Each bar represents the mean of two experimental values (standard errors did not exceed 5%)

We further examined the ability of the photoaffinity probe to label cytochrome P450 2B1 in microsomes prepared from phenobarbital (P450 2B1 inductor) treated rats Microsomes, vesicles of endoplasmic reticulum membranes, were used for labelling of P450 2B1 since the microsomes represent a membrane-enzyme system closely corresponding to "*in vivo*" conditions, contrary to purified P450 released from membranes As mentioned above, experiments were conducted with DIA and vit E in DLPC suspensions allowing to determine the decrease of labelling caused by the perturbants only Since microsomes are a complex mixture of various proteins, labelled P450 2B1 had to be separated after the irradiation step by SDS-electrophoresis Then, a gel strip containing the sample track was cut into 4 mm slices and the radioactivity of the slices, corresponding to the P450 2B1 standard, was measured by scintillation counting The radioactivity found in the gel is illustrated in Fig 5 Again, DIA caused a drop in P450 2B1 labelling the probe incorporation was blocked by 49% However, vit E, unlike the previous experiments with pure P450 enzyme, failed to affect the labelling These differences are most probably accounted for by a high lipid and protein content of microsomal

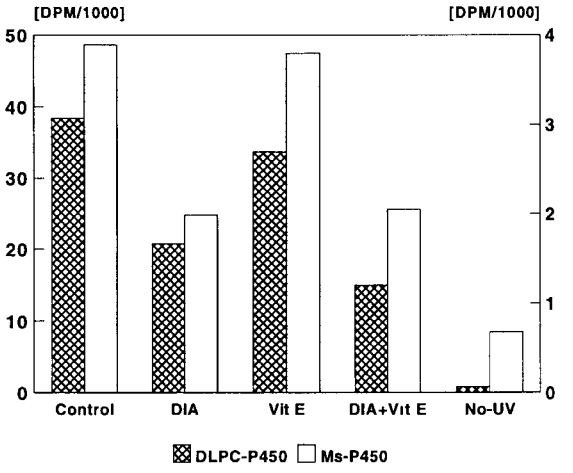

Figure 5. Photoaffinity labelling of cytochrome P450 2B1 in **DLPC** suspensions or microsomes (**Ms**) with 9 μmol/l [^{3}H]DIA-N_2 in the presence of 20 molar excess of perturbants **Control**, no perturbant, **DIA**, diamantane, **Vit.E**, vitamin E, **DIA+Vit.E**, diamantane + vit E, **No-UV**, no photolysis For DLPC-P450 and Ms-P450 the left and right y-axis, respectively, are used In the case of Ms-P450 the radioactivity of P450 was determined in acrylamide gel slices after electrophoretic separation of microsomal proteins Data are means of three (DLPC system) or two (Ms system) parallel experiments (the standard errors did not exceed 10%)

samples which served as natural scavengers of the photoactivated probe Thus, the scavenging effect of vit E on the probe binding outside the P450 active site was nil

Table 4. Yield of photoaffinity probe incorporation in different systems

System	Radioactivity of photoaffinity probe [DPM/mg protein]		Average yield [%]
	Added	Found	
BSA	87,818 ± 2786	10,430 ± 870	11 8
P450	84,364 ± 5112	9125 ± 349	10 8
P450+DLPC	218,455 ± 997	38,393 ± 2075	17 6
Microsomes*	6,074,900 ± 160418	86,400 ± 2743	1 4

* Radioactivity was calculated based on P450 2B1 specific content in microsomes (2 1 nmol P450/mg of microsomal protein) and the relative molecular weight of 50,000 Data are means of two parallel experiments ± standard errors

Data in Table 4 summarize the probe labelling efficiency in the different systems used. The concentration of the probe employed in all experiments was approximately 5 times the value of the spectral dissociation constant (K_s = 3.4 μmol/l) determined for the probe with purified P450 2B1. The yields of the probe incorporation to BSA or P450 2B1 in water solutions reflected the similar ability of both proteins to bind the probe to their hydrophobic binding sites. A comparison of the results of experiments with P450 2B1 dissolved in the absence or presence of DLPC suspensions clearly suggests that the DLPC system enhances the label incorporation. This observation is most likely a consequence of the DLPC influence on the P450 binding affinity for the probe. As it is well documented P450 reconstituted in artificial lipids or in microsomal preparations displays enhanced binding affinity for substrates (Yun et al. 1997) compared to purified ones. In addition, DLPC might increase the probe availability since the concentration of hydrophobic compounds in DLPC, replacing the membrane environment of P450, is assumed to be higher than that in water solution. On the other hand, P450 labelling in microsomal preparations was much weaker than in other systems. This about 10-fold reduction of the probe incorporation was most likely caused by excess of proteins and microsomal lipids. In this regard it should be emphasized that cytochrome P450 2B1 comprises a rather small fraction (< 10%) of the total microsomal proteins which might compete for the probe incorporation. 2-Aziadamantane, a similar compound as tested here,was successfully used for labelling of membrane spanning protein regions (Bayley and Knowles 1980). In addition, microsomal lipids, was used as the "solvent" of hydrophobic 3-azidiamantane, lower its actual concentration and thus its availability for binding to the active centre and/or serve as additional targets of the probe incorporation. Since no potential non-specific labelling occur in microsomes without any need for additional scavengers (no influence of vit. E added) the probe is probably also incorporated into other molecules present in the microsomes.

The molar fraction of radiolabelled cytochrome P450 2B1 was calculated based on the probe specific radioactivity (isotopically diluted) to be approximately 0.13; this means that 13% of P450 molecules in the reaction mixture were covalently modified with the probe. Similar results were obtained with non-radioactive 3-azidiamantane the incorporation of which (after photoactivation) was examined by means of difference spectroscopy (Hodek et al. 1997). The loss of the substrate binding capacity, reflecting the probe incorporation, was found to be 12%.

Taking all the probe characteristics together, 3-azidiamantane belongs among the well applicable photoaffinity probes for the identification of the cytochrome P450 2B1 binding site region.

In summary, tests with photoaffinity labelling of cytochrome P450 2B1 proved that 3-azidiamantane is a useful tool for P450 2B1 active centre studies. This conclusion is drawn based on the high specificity of the probe labelling shown in

experiments with various scavengers and P450 substrates Moreover, the probe is readily photolyzed by long-wavelength UV-light producing mainly short-lived highly reactive intermediates essential for the labelling of the hydrophobic active centre of cytochrome P450 2B1 Experiments with radiolabelled 3-azidiamantane for the identification of active centre amino acids are in progress

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