Sci Pharm. 2008; 76: 241–257 doi:10.3797/scipharm.0803-01

Catalytic Activity of Cytochrome P-450 using $NADP^+$ Reduced by an Anionic Hydride Organosiloxane

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Abstract

Cytochrome P-450 (P450) catalyzes a wide variety of chemical reactions; however, its use for *in vitro* assays has several limitations, the most striking one is the use of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) coenzyme. In this work, the P450 activity using $NADP^+$ reduced by an anionic organosiloxane, commonly named silica hydride, was evaluated. The results showed that the reduction of $NADP^+$ with silica hydride was concentration- and time-dependent. P-450 activity was maintained when $NADP^+$ and silica hydride were added during the reaction; however, it was lower than when commercial NADPH was employed. This is due to the ability of silica hydride to reduce P450

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iron atom as corroborated by the electronic paramagnetic resonance (EPR). Furthermore, this compound possibly chelates Fe^{II} because, in its presence, the P450 affinity for aniline diminishes. However, the P450 activity was the best when $NADP^+$ was reduced by silica hydride before the former was added to the reaction. Therefore, this system could be apt for studying biotransformation reactions.

Keywords

Cytochrome P450 • Drug metabolism • Electron paramagnetic resonance • Nicotinamide adenine dinucleotide phosphate • Silica hydride

Introduction

Cytochrome P450 (P450) enzymes belong to a family of monooxygenase proteins, which catalyze a wide variety of oxidative reactions such as hydroxylation of aliphatic and aromatic carbons, N- and O-dealkylation, S-oxidation and epoxidation of olefins [1, 2]. Microsomal P450 enzymes need two electrons during their catalytic cycle [3]. The first electron is transferred from reduced nicotinamide adenine dinucleotide phosphate (NADPH) by NADPH-P450 reductase, converting the ferric iron atom of the heme group to its ferrous state. The second electron, followed by a proton, is transferred to the ferrous-dioxy species to form an iron-hydroperoxo (Fe^{III}-OOH) intermediate [4]. This electron may come from either NADPH-P450 reductase or from cytochrome b_5 [5, 6].

Microsomal P450 enzymes are important during the biosynthesis of many endogenous compounds, the biotransformation of xenobiotics, and could play an important role in the bioremediation processes as well as in organic catalysis [7–9]. However, the use of P450 enzymes is limited because of the NADPH requirements for supplying electrons during their catalytic cycle. In this sense, several studies have been carried out to obtain a source of reduced $NADP^+$ for the P450 reactions. For instance, a regenerator system using glucose-6-phosphate dehydrogenase has been used [10, 11].

Recently, several researchers have observed that an anionic hydride organosiloxane, commonly named silica hydride, acts as an electron source through interstitially embedded hydrogen anions (hydride ions, H^- ions) [12]. This compound has presented interesting biochemical properties *in vitro,* including neutralization of reactive oxygen species (ROS), such as super oxide anion, singlet oxygen, and hydroxyl radical, among others [13, 14]. In addition, it acts as a catalyst for redox reactions, such as in the NAD^+ reduction, which implies the electron transfer from silica hydride to NAD^+ [15, 16]. The electron transfer reactions can be studied by electron paramagnetic resonance (EPR) [17]. This technique identifies free radicals, transition metals, and uncoupled electrons during the catalytic cycle of P450 [18].

The aim of this work is to study the oxidation reaction of aniline catalyzed by P450 from rabbit liver using $NADP^+$ reduced by silica hydride, in order to find an alternative system with which the biotransformation reactions of several compounds can be carried out.

Results and Discussion

Silica hydride characterization

The ^1H NMR spectrum was recorded in a Varian Mercury 300 spectrometer, using D_2O as solvent. Si-OH protons appear as a multiplet centered at 2.41 ppm, which integrates for 16.5 H-atoms; a broad signal for one H-atom centered at 1.6 ppm was assigned to $–SiH_2–$ moiety, and a quarter with a coupling constant of 81 Hz and centered at –0.31 ppm was assigned to six hydrides (Fig. 1), in agreement with the reported values [12].

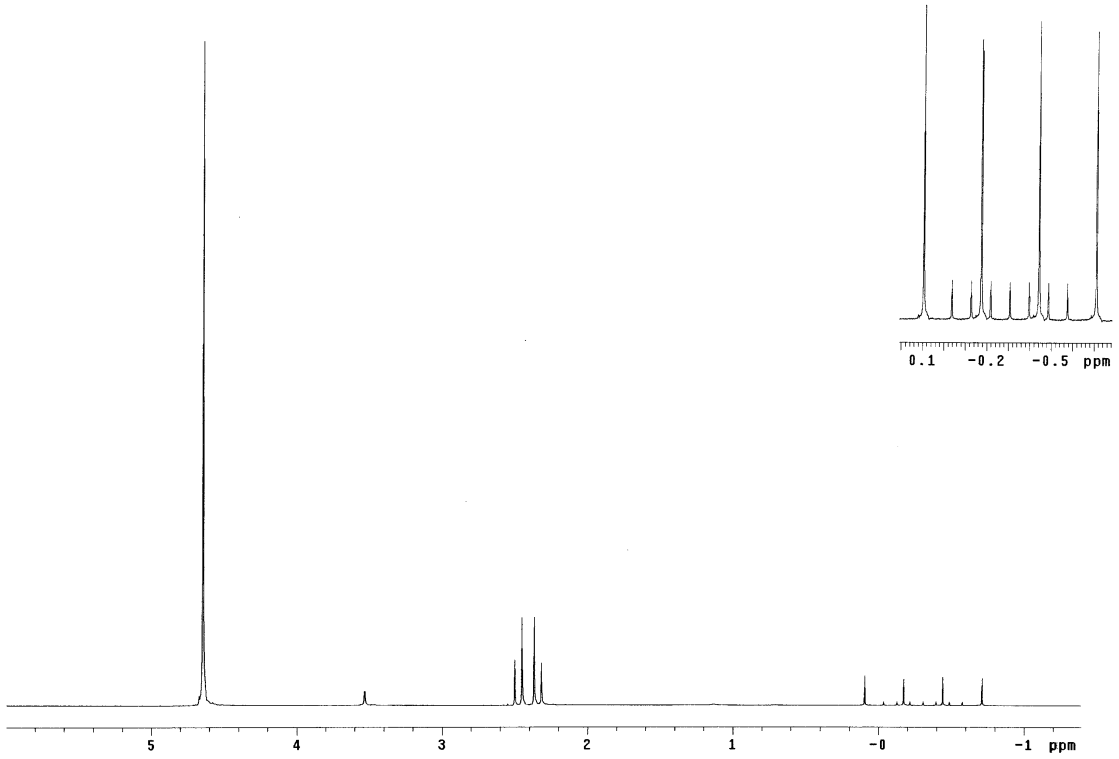

Fig. 1. ^{1}H-NMR spectrum of silica hydride. A Varian Mercury 300 MHz nuclear magnetic resonance spectrometer was used as one of the means to identify the siliceous hydride compound.

Reduction of NADP^{+} by silica hydride

The results showed that NADP^{+} was reduced by silica hydride, supporting the earlier reports in which this compound was able to reduce NAD^{+} to NADH [15, 16]. It is important to mention that both NAD^{+} and NADP^{+} have the same reduction potential (−0.320 V) [19]; hence, both coenzymes can be reduced by silica hydride, which has a reduction potential of −0.862 V [16]. Furthermore, the reduction of NADP^{+} was studied with different concentrations of silica hydride (0.29, 0.57, 1.15, 2.3, 4.6, 9.2, 18.4, and 36.8 mg/mL) and different reaction times (20, 40, and 60 min), depending it of both variables. Fig. 2, shows that when the silica hydride concentration is increased (2.3, 4.6, and 9.2 mg/mL), the amount of NADPH is greater, as can be seen with the different absorbance values of NADPH at 340 nm.

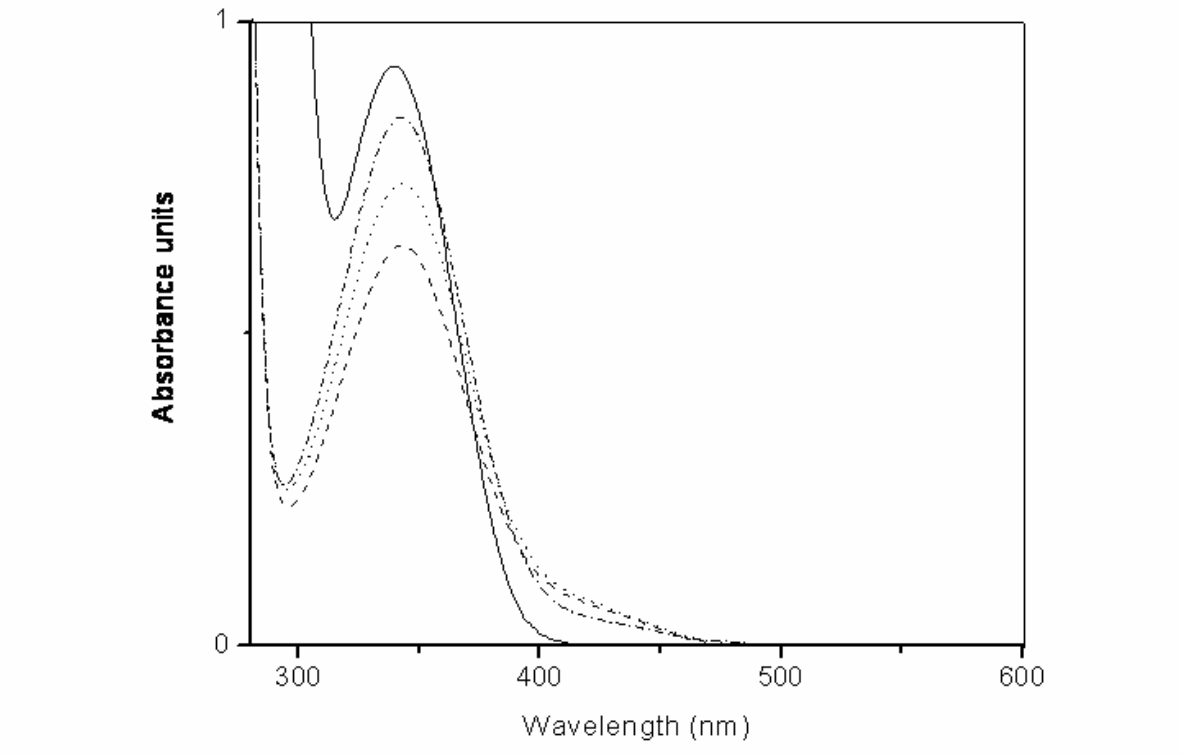

Fig. 2. UV/Vis spectra of $NADP^+$ reduced by using different concentrations of silica hydride: _____ 2.3, _____ 4.6, _ _ _ 9.2 mg/mL, and ____ NADPH (0.3 mM).

However, the latter compound at 18.4 and 36.8 mg/mL was slightly soluble, and with the addition of $NADP^+$, the reaction produced a lot of gas. Therefore, a concentration between 4.6 and 9.2 mg/mL of silica hydride was chosen for the kinetic experiments, finally using.5.7 mg/mL.

With respect to time, the amount of NADPH generated is greater after 20 min, because it has been reported that the reduction potential of silica hydride increases when it is added to the solution, from −0.322 V to −0.862 V after 23 min. The amount of reduced $NADP^+$ at 40 and 60 min was very similar to that at 20 min. In Fig. 3, it is possible to observe that there is not difference between the amount of NADPH produced after 20 and 40 min at different concentrations of silica hydride.

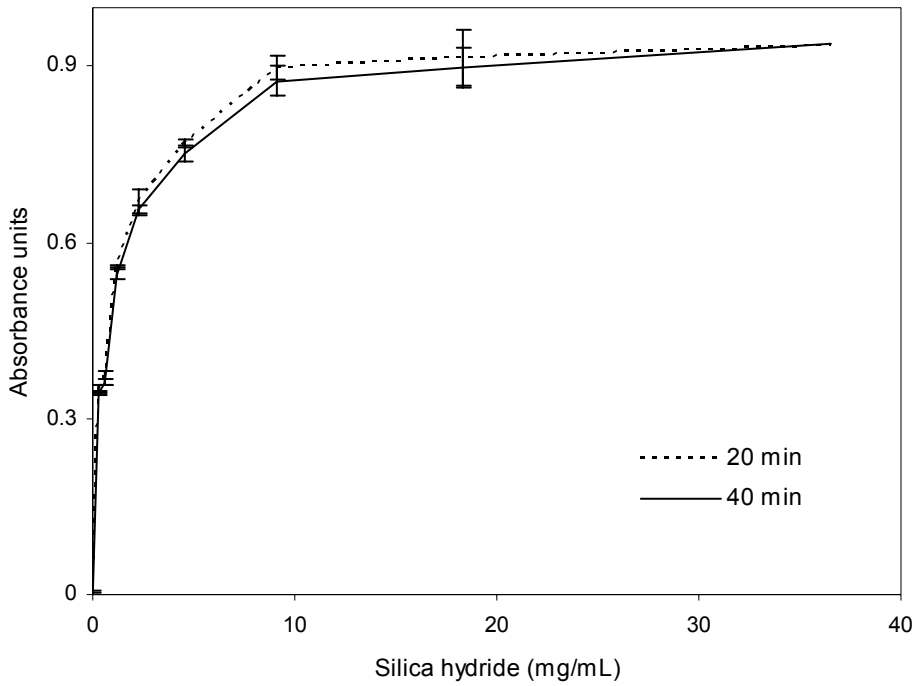

Fig. 3. $NADP^+$ reduced with different concentrations of silica hydride and measurement at 340 nm after 20 and 40 min of addition of silica hydride; the data are expressed as mean (n=3) individual preparations.

This could be because, at this time, silica hydride reduction potential is at maximum (−0.862 V) [16]. Furthermore, it has been demonstrated that its reduction potential is maintained for extended periods at neutral pH, in addition with the fact that the release of H^- ions and the experiments were carried out at pH 7.4. Therefore, it has been concluded that the pH has a significant impact on the efficacy of silica hydride as a reducing agent [13, 14].

Determination of P450 activity

The P450 activity was measured by the aniline oxidation to *p*-aminophenol. P450 showed catalytic activity when $NADP^+$ and silica hydride were added to the reaction system; however, its activity was lower than when commercial NADPH was used (Fig. 4).

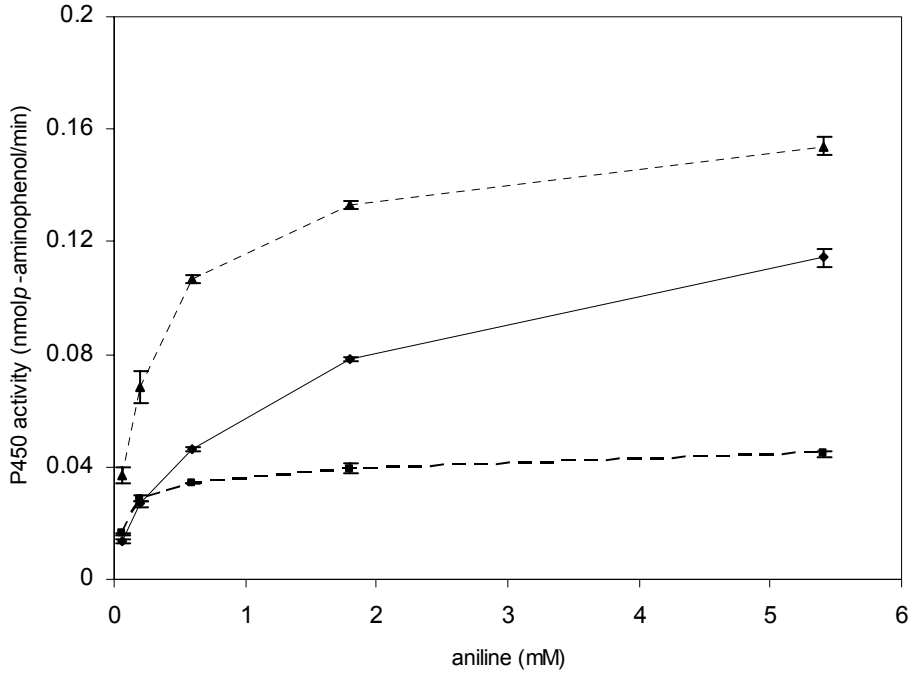

Fig. 4. Catalytic activity of P450 using: - - - - - - silica hydride and $NADP^+$ during the reaction of P450 (**A**), ——— commercial NADPH (0.3 mM) (**B**), and **…..** silica hydride and $NADP^+$ mixed before adding to the reaction (**C**). The data are expressed as mean (n=3) individual preparations.

The loss of P450 activity could be due to the fact that silica hydride was able to reduce the $NADP^+$ and the heme iron atom of P450 at the same time, because their reduction potentials are very similar.

P450 with and without substrate has a reduction potential of −0.225 and −0.300 V, respectively, whereas for $NADP^+$ it is −0.320 V [2, 20]. Therefore, if silica hydride reduced the iron atom from its ferric (Fe^{III}) to ferrous (Fe^{II}) state, it is possible that the catalytic activity of P450 diminishes due to the low affinity of aniline for Fe^{II} [21]. This effect could be due to the fact that during the P450 catalysis the Fe^{III} has high LOMO energies, which allow it to carry out an electrophilic attack on aniline [22]. To explore this phenomenon, docking studies were done to evaluate the affinity between aniline and CYP2B4, an isoform of P450 with its iron atom in two oxidation states, Fe^{II} and Fe^{III}. These results showed that the affinity for aniline of this enzyme, with both oxidation states of the iron atom,

was very similar (Tab. 1). Therefore, it is possible that silica hydride acts as an inhibitor of P450.

Tab. 1. Affinity values of CYP2B4 with its iron atom as Fe^{II} and Fe^{III} for aniline obtained by computational docking

	ΔG (kcal/mol)	K_d **(nM)**
CYP2B4 (Fe^{II})	–12.14	1.26
CYP2B4 (Fe^{III})	–12.25	1.05

To evaluate this possibility, several experiments were done. In the first experiment, P450 and silica hydride were mixed and immediately added to the reaction. In the second one, P450 and silica hydride were mixed for 20 min because it is the time of the reaction and also the time when silica hydride increases its reduction potential. The results showed that in the first experiment, the P450 activity was not modified in comparison with P450 plus NADPH. On the other hand, the result of the second experiment showed that the P450 activity diminished when $NADP^+$ and silica hydride were added to the reaction at the same time. To corroborate that P450 lost its activity when silica hydride is added to the reaction, a third experiment was carried out using NADPH and silica hydride in the reaction. The results showed that the P450 activity decreased, in relation to the same system but without the compound.

Therefore, the addition of silica hydride during the reaction diminishes the catalytic activity of P450, which could have several explanations: (1) silica hydride might inhibit the catalytic activity by binding itself to the substrate P450 binding site; (2) the decreased catalytic activity may be a result of an increased enzyme destruction or denaturation associated with silica hydride; (3) this compound could chelate the Fe^{II} and block the interaction of the iron with aniline. Taking into account the last possibility, the process could depend on the elapsed time because the loss of P450 activity was observed in the second and the third experiments when silica hydride and the enzyme were mixed for 20 min. Also, the loss of P450 activity when

this compound was added during the reaction could be due to the production of scavenger radicals during the catalytic cycle, because silica hydride was able to reduce the ROS [13, 14], such as the superoxide anion, which was formed during step 3 when the oxygen was bound to ferrous P450 [2]. Consequently, the addition of silica hydride during the P450 reaction does not prove to be a good system; for this reason the experiments were carried out by reducing the $NADP^+$ with silica hydride before it was added to the reaction.

Both compounds were mixed for 30 min; when this time elapsed the mixture was added to the reaction system, which contained P450 and aniline. The obtained results showed that the P450 activity was better with this system than when silica hydride and $NADP^+$ were separately added to the reaction (Fig. 4) and than when commercial NADPH was used. As can be seen in Tab. 2, with the former system the Vmax of P450 increased and its K_M diminished showing the best affinity for aniline in relation to the latter system in which the V_{max} diminished and its K_M increased presenting, inclusive, low affinity for aniline in comparison with that obtained with commercial NADPH. However, the biotransformation reactions with $NADP^+$ reduced by silica hydride before the reaction imply two steps: the reduction and the addition of the generated NADPH to the reaction. It could be a good system for evaluating in vitro the biotransformation of several compounds because it proves to be cheaper than using glucose 6-phosphate dehydrogenase and, in this way, other studies can be carried out to design a silica hydride column for the regeneration of NADPH obtained from the reduction of $NADP^+$.

Tab. 2. K_M and V_{max} of P450 activity using NADPH or $NADP^+$ reduced with silica hydride

Source of electrons	V_{max} (mM min^{-1})	K_M (mM)
$NADP^+$ + silica hydride [a]	0.053 ± 0.002	0.88 ± 0.049
$NADP^+$ + silica hydride [b]	0.135 ± 0.003	0.201 ± 0.040
NADPH	0.08 ± 0.002	0.357 ± 0.056

[a] $NADP^+$ and silica hydride were added during the reaction.

[b] $NADP^+$ was reduced with silica hydride before its addition to the reaction.

EPR measurements

The iron atom reduction with silica hydride was corroborated by EPR studies. First results with respect to the enzyme were obtained from microsomes. Fig. 5a shows a single rhombic EPR line with *g* tensor at g_x = 2.4205, g_y = 2.2442, and g_z = 1.9165, characteristic of low spin iron (*s* = 1/2) from P450. After the addition of silica hydride to microsomes, the signal from the iron was not observed, which indicates that the iron was reduced to Fe^{II} (Fig. 5b). These results are in accordance with previous reports about the reduction of iron from cytochrome *c* with silica hydride [13].

Other EPR experiments were done with the complete reaction system (P450, $NADP^+$, silica hydride, and aniline). The EPR experiment was carried out 15 min after the reaction started. The obtained EPR spectrum did not show any signal, which could be due tlo the fact that when the reaction is realized the Fe^{III} is reduced to Fe^{II} (Fig. 5c); therefore, silica hydride reduces the iron and possibly chelates the Fe^{II}.

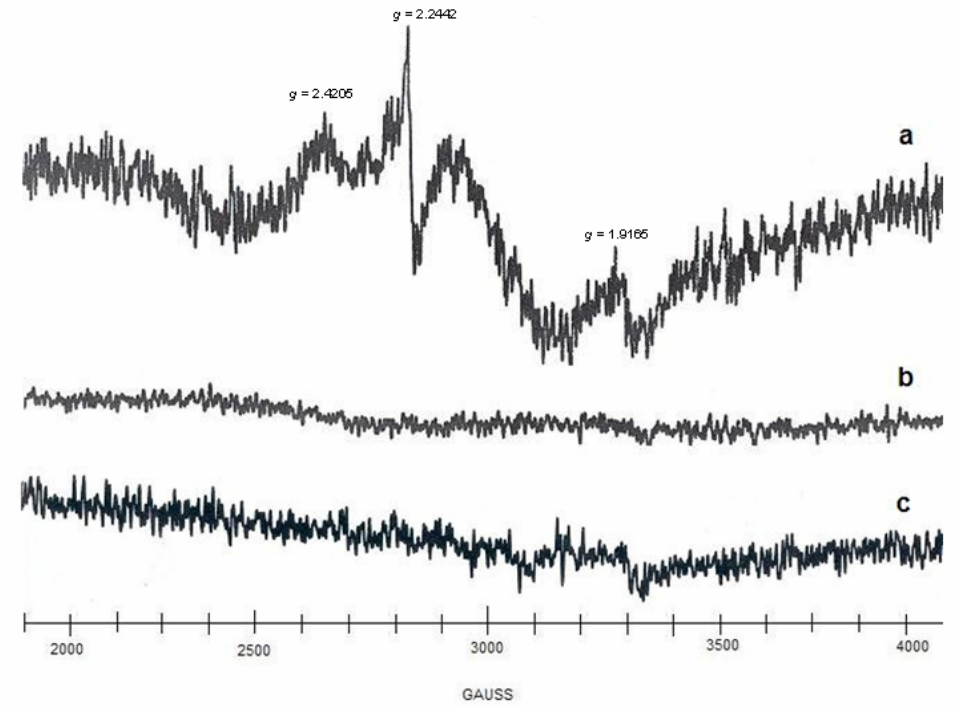

Fig. 5. EPR spectra of (a) microsomes at 95 K, (b) microsomes reduced with silica hydride, and (c) the complete system reaction 15 min after starting.

Furthermore, in this spectrum it was not possible to observe any signal from an amino acid such as Tyr or Trp, because the experiments were done with nitrogen instead of helium. Therefore, other experiments could be carried out with helium at different reaction times.

Experimental

Chemicals

All chemicals employed were of reagent grade obtained from Sigma–Aldrich. Aniline was redistilled before be used.

Synthesis of silica hydride

Silica hydride was synthesized based on previous reports [12]. NMR identified the product obtained in D_2O. The ^1H NMR spectrum was recorded on a Varian Mercury 300 (^1H, 300.08 MHz) following standard techniques.

Preparation of liver microsomes

Male New Zealand rabbits weighing 2.25 ± 0.25 kg were used as a source of microsomes for all experiments. The rabbits were fasted for 48 h and then killed. Liver microsomes were prepared and washed as was previously described [23]. The washed microsomes were suspended in potassium phosphate buffer 0.1 M and pH 7.4 with 20% of glycerol. P450 was determined from the CO-difference spectrum as was reported by Omura and Sato [24] and thereafter the samples were stored at −80ºC.

Reduction of $NADP^+$ by silica hydride

$NADP^+$ 0.5 mM in potassium phosphate buffer 0.1 mM pH 7.4 was reduced using different concentrations of silica hydride (from 0.29 to 36.8 mg/mL) and the formed NADPH was measured at 20, 40, and 60 min. A UV–Vis spectrometer (Perkin Elmer model Lambda 25) was used to analyze the $NADP^+$ reduction by observing an increase in the absorbance at 340 nm.

Determination of P450 activity

The reaction mixture for the oxidation of aniline was achieved by using 1 nmol of P450, 5.7 mg/mL of silica hydride (this concentration was previously identified as the optimum), and $NADP^+$ or NADPH 1 mM (when it was used), in a final volume of 0.70 mL of potassium phosphate buffer 0.1 M pH 7.4. The concentration range of aniline used for the kinetic experiments was from 0.4 to 6 mM.

The reaction was stopped after 30 min by adding trichloroacetic acid, reaching a final concentration of 7.5%. The reaction mixture was centrifuged at 5,000 rpm and, after that, the *p*-aminophenol determination was carried out following existing literature [25,26]. A volume of 0.25 mL of sodium carbonate 20% (p/v) and 0.75 mL of phenol 2% (p/v) were added to 0.5 mL of the supernatant and incubated at 37 ºC for 30 min. The colored product was measured at 630 nm. For the reaction, three blanks were made, (1) without enzyme, (2) without silica hydride, and (3) without $NADP^+$ to confirm that the aniline oxidation was carried out only when all reactants and the enzyme were together.

EPR measurements

EPR measurements were realized in an X-Band frequency (9.2 GHz) with a JES-RES 3X JEOL spectrometer, operating at 100-kHz field modulation. The EPR X-band spectra were recorded at 77 K. The *g* values were calculated from measurements of the magnetic field with a resolution of ± 0.01 mT as well as from microwave frequency parameters and the DPPH marker.

Molecular modeling

The optimized energy of aniline was obtained by using DFT calculations at B3LYP/6-31G* level in a Gaussian 98 software [27].

To identify the recognition mechanism and affinities between CYP2B4 (as Fe^{II} and Fe^{III}) and aniline, docking simulations were done on the 3-D structure of CYP2B4 from rabbit (PDB code: 2bdm) [28]. Before starting the docking evaluations, the partial atomic charges (Gasteiger-Marsili formalism), all possible rotable bonds of the aniline and the Kollman charges for all atoms in both CYP2B4

(with Fe^{II} and Fe^{III}) were assigned by using the AutoDock Tools 1.4.5 [29]. Moreover, missing residues were built and hydrogen atoms were added to the amino acids of the protein with the same program. The iron charges (Fe^{II} and Fe^{III}) were manually assigned.

For the docking studies, the latest version of AutoDock 4.0.1 program [29] was chosen because its algorithm allows full flexibility of small ligands and residues. In this case, we have selected those residues involved in the ligand recognition. The input preparation of the protein and the ligand structures as well as the definition of the binding sites was carried out under a GRID-based procedure [30]. First, a rectangular grid box was constructed over all protein (126 x 126 x 126 $Å^3$) with grid points separated by 0.375 Å.

All docking simulations were done with the hybrid Lamarckian genetic algorithm, with an initial population of 100 individuals randomly placed and a maximum number of energy evaluations (1.0×10^7). The obtained docked orientations within a root-mean square deviation of 0.5 Å were clustered together. The lowest energy cluster, returned for each compound by AutoDock, was used for further analysis. All other parameters were maintained at their default settings.

All the results of the docking visualizations were achieved by using a Visual Molecular Dynamics (VMD) program [31].

Data analysis

Each assay was performed by triplicate and the results were expressed as mean ± SD. The kinetic results were fit to a Michaelis-Menten model and the kinetic parameters were estimated by Lineweaver Burk method using origin 6.1 program.

Acknowledgements

The authors thank CONACYT (62488) and COFAA-SIP/IPN (20080026) for financial support, Dr. Juan Manuel Aceves for the synthesis of silica hydride, and Dr. Itzia Padilla for silica hydride ^1H NMR measurements and descriptions.

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Received March 13th, 2008
Accepted (after revision) April 16th, 2008
Available online at www.scipharm.at April 21st, 2008