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for

Replacement of the Natural Cofactors by Selected Hydrogen Peroxide Donors or Organic Peroxides Results in Improved Activity for CYP3A4 and CYP2D6

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Chemicals

The CYP3A4 pSE3A4His expression plasmid was a gift from Dr. J. R. Halpert from the University of Arizona, the CYP2D6 expression plasmid (DB6 with His_5 and M374V) was obtained from Dr. F. P. Guengerich from Vanderbilt University, and the cytochrome P450 reductase OR263 plasmid was kindly donated to us by Dr. Charles B. Kasper from the University of Wisconsin. The culture media ingredients yeast extract, tryptone, peptone, as well as the CYP2D6 substrate AMMC (3-[2-(*N,N*-diethyl-*N*-methylammonium)-ethyl]-7-methoxy-4-methylcoumarin) and the product AHMC (3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride) were purchased from BD Biosciences (San Jose, CA). DH5a supercompetent cells and agarose were from Invitrogen (Carlsbad, CA). The 2',5'-ADP Sepharose 4B and DEAE Sepharose resins were from Amersham Bioscience (Baie d'Urfé, QC, Canada). The His-Select™ Nickel Affinity Gel was purchased from Sigma (St. Louis, US). The CYP3A4 substrate testosterone (4-androsten-17β-ol-3-one) was a king gift from Dr. Eisenberg in our department and the metabolites 6β-hydroxytestosterone (4-androsten-6β,17β-diol-3-one) and 6α-hydroxytestosterone (4-

androsten-6α,17β-diol-3-one) were purchased from Steraloids (Newport, RI). All solvents were purchased from Fisher and were of HPLC grade. All chemicals were used without further purification. Water was obtained from a Milli-Q Synthesis (Millipore, San Jose, CA) filtration system. All other chemicals were purchased from Sigma or Aldrich.

Instruments

UV absorption spectra were recorded on a Cary 5000 UV spectrophotometer (Varian, Mississauga, ON, Canada). Fluorescence measurements were obtained on a Spectramax GeminiXS (Molecular Devices Corp., Sunnyvale, CA) using 96-well flat bottom assay plate (Corning Incorporated Life Sciences, New York, NY). Analytical HPLC analyses were performed on an Agilent 1100 modular system consisting of an auto-sampler, a quaternary pump system, a photodiode-array detector, a fluorescence detector, and a thermostated column compartment. The Agilent Chemstation software version A.10.02 was used to control the operation and data acquisition. Analysis of dextromethorphan *O*-demethylation by CYP2D6 was carried out using a Synergi 4 µm Hydro-RP 80 Å column with mobile phase A (0.05% TFA in water) and B (100% acetonitrile) at a flow rate of 1 mL/min. The elution gradient was from 20% to 90% B over 6 min, then held for 2 min. Fluorescence ($?_{ex}$ = 280 nm, $?_{em}$ = 310 nm) was used to monitor the elution. The retention times for dextromethorphan and dextrorphan were 5.4 and 6.3 min respectively. Analysis of testosterone 6β-hydroxylation by CYP3A4 used a 150 x 4.6 mm Zorbax Eclipse XDB-C8 5µm column from Agilent protected by an analytical guard column. The elution consisted of a first isocratic step at 15% acetonitrile in water for 4 min, before a linear gradient to 50% acetonitrile over 12 min. The flow rate was 1.5 mL/min, and the column temperature was set to 30°C. Detection was at 244 nm. Under these conditions, the retention times of testosterone, 6β-hydroxytestosterone, 6α-hydroxytestosterone and cortexolone were 16.3, 9.9, 10.5 and 14.2 min respectively.

Expression and purification of His-tagged CYP2D6

The CYP2D6 plasmid was transformed into *E. coli* DH5a supercompetent cells and grown overnight at 37ºC on Luria-Bertani (LB) agar plates with ampicillin (100 mg/L). LB media consisted of: 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter, autoclaved for 20 min at 121°C. One separate colony was added to Terrific Broth (TB) medium (5 mL prepared from: 12 g tryptone, 24 g yeast extract, 4 mL glycerol, 2.31 g KH_2PO_4,

12.54 g K_2HPO_4 per liter, autoclaved) containing ampicillin (100 mg/L). After incubation (300 rpm, 37°C, 17 h), a portion of this culture (2 mL) was diluted in TB (100 mL) containing ampicillin (100 mg/L), potassium dihydrogen phosphate (0.23 g) and potassium hydrogen phosphate (1.25 g). After a second incubation (180 rpm, 33°C, 5 h), this mixture was further diluted in TB (10 mL in 500 mL) containing ampicillin (100 mg/L), trace elements solution (150 µL), potassium dihydrogen phosphate (1.1 g), and potassium hydrogen phosphate (5.23 g). The trace elements solution consisted of 100 mM $FeCl_3$, 10 mM $ZnCl_3$, 10 mM Na_2MoO_4, 14.3 mM $CaCl_2$, 7.4 mM $CuCl_2$, 10 mM H_3BO_3 in 1 mL of concentrated HCl + 9 mL of MilliQ water. The culture was fermented for 30 min at 33°C and 250 rpm. Next, after addition of the heme precursor δ-aminolevulinic acid (δ–ALA, 8.4 mg/L), thiamine (0.68 g/L) and quinidine (0.03 mM), the culture was incubated at 37°C and 180 rpm until OD_{600} 0.7-0.8 (~2.5 h). Protein expression was induced with β-D-thiogalactoside (IPTG, 0.5 mM) and the culture was further grown at 25°C and 150 rpm for 48 h.

The cells were harvested by centrifugation (4000 *g*, 25 min), and the harvested cells (100 g) were suspended in TES buffer (12 mL/gram cells of: 500 mM Tris acetate at pH 7.5, 250 mM sucrose, 0.25 mM EDTA), followed by addition of lysozyme (3 mg/g cells). The same volume of pre-cooled water was added and gently stirred for 30 min at 4°C before centrifugation (3393 *g*, 4°C, 15 min). The pellets were resuspended in sonication buffer (30 mL of: 0.1 M potassium phosphate buffer at pH 7.4 containing 20% glycerol (*v/v*) and 6 mM magnesium acetate). The cells were further lyzed by sonication (7 times at 60% duty cycle and power 8 for 20 s each time) after addition of leupeptin (1 µg/mL), aprotinin (0.04 µg/mL), bestatin (1 µM) and ß-mercaptoethanol (20 mM). A salt-ice bath was applied to keep the mixture cool. Following centrifugation (10 000 *g* for 20 min and 75 000 *g* for 60 min at 4°C), the supernatant was loaded onto the nickel affinity column (1 mL) equilibrated with buffer C (20 mM potassium phosphate buffer at pH 7.4 containing 20% glycerol, 2 g/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.5 M potassium chloride), and buffer D (buffer C containing 10 mM ß-mercaptoethanol). The column was washed with buffer D (10 mL) and buffer E (10 mL of: 20 mM potassium phosphate buffer at pH 7.4 containing 20% glycerol (*v/v*), 0.5 M potassium chloride, and 10 mM ß-mercaptoethanol). The protein was eluted

with buffer E without ß-mercaptoethanol but containing imidazole (200 mM). The colored fractions were pooled and dialyzed for 4 h against buffer F (2 × 4 L of 10 mM potassium phosphate buffer at pH 7.4 containing 0.13 mM EDTA and 0.1 mM DTT). The fractions containing 50 kDa proteins were identified by SDS-PAGE (Homogeneous 12.5%). The overall yield was calculated to be 0.563 mg/L.

Expression and purification of His-tagged CYP3A4

CYP3A4 was expressed using a modification of the procedure used by Domanski *et al.*[1] The plasmid was transformed into *Escherichia coli* DH5α supercompetent cells, and the cells grown overnight on LB agar plates with ampicillin (50 μg/mL). Culture tubes containing LB media supplemented with ampicillin (50 μg/mL) were inoculated next. After 24 h of growth at 37°C with shaking at 250 rpm, 10 flasks of 750 mL TB medium containing ampicillin (50 μg/mL) were inoculated each with 3 intermediate culture tubes. The flasks were placed at 37°C with shaking at 250 rpm for 2 h (i.e. until OD_{600} = 0.6), after which protein expression was induced with IPTG (1 mM) followed by addition of δ-ALA (80 mg/mL). The incubation temperature was reduced to 30°C, with shaking set to 190 rpm for 48 h. The cells were collected by centrifugation at 4000 *g* and 4°C for 15 min. The pellets were resuspended in buffer A (50 mL of: 100 mM 3-[*N*-morpholino]propanesulfonic acid] (MOPS) at pH 8 containing 10% glycerol, 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride (PMSF)). All subsequent procedures were performed at 4°C. Lysozyme (10 mg/g of cells) was added, and the suspension was stirred slowly for 15 min, after which the suspension was supplemented with protease inhibitors (1.6 μg/mL leupeptin, 1 μg/mL aprotinin, 0.8 μg/mL bestatin, and 0.7 μg/mL pepstatin A). The suspension was then sonicated on an ice-salt bath at 60% and power 8. After 2 sonication cycles of 20 s each, the suspension was centrifuged at 100 000 *g* for 60 min. The supernatant was removed and the pellets resuspended in buffer B (10 mL of: 100 mM MOPS at pH 7.4, containing 10% glycerol and 2 mM PMSF). CHAPS (0.5%) and potassium chloride (0.5 M) were then added. Cells were homogenized and gently stirred for 2 h before centrifugation (1 h at 100 000 *g*). This CHAPS-solubilized P450 preparation was then stored at -80°C until the next step.

The His-tagged protein was purified by affinity column chromatography using the Ni-NTA metal affinity resin (1.5 mL), pre-equilibrated with 5 bed volumes of EQ-buffer

(buffer B containing 0.5% CHAPS, 0.5 M KCl, and 5 mM imidazole) at a flow rate of 0.5 mL/min. The P450 preparation was then loaded onto the column at the same flow rate. The column was washed with 10 bed volumes of EQ-buffer, then with 10 bed volumes of 100 mM MOPS buffer at pH 7.4 containing 10% glycerol and 10 mM imidazole. The P450 was eluted in EL-buffer (100 mM MOPS at pH 7.4 containing 10% glycerol and 200 mM imidazole). The orange-colored fractions were compared spectroscopically for protein content and by SDS-PAGE (12.5% homogeneous gels) for purity assessment. The fractions containing the CYP3A4 protein were pooled and dialyzed (twice 4 L of: 100 mM MOPS buffer at pH 7.4 containing 10% glycerol, 0.2 mM dithiothreitol and 1 mM EDTA) at 4°C. The sample was aliquoted and stored at -80°C. The overall yield was 0.6 mg per liter of culture.

Quantification of P450 enzymes

The P450 content was measured by reduced carbon monoxide difference spectra following the method described by Omura and Sato[2]. Thus heme was reduced by addition of a few solid grains of sodium dithionite ($Na_2S_2O_4$) in quartz cuvettes containing the buffered enzyme solution, and formation of the enzyme-carbon monoxide complex was achieved by slow bubbling of CO gas into the reduced enzyme solution for about 20 s. The UV absorption spectra was recorded, and the concentration of P450 was determined using the extinction coefficient $e_{450} = 91\ mM^{-1}\ cm^{-1}$.

Cytochrome P450 reductase (CPR) expression and purification

E. coli DH5a supercompetent cells were transformed with the cytochrome P450 reductase OR263 plasmid and spread onto LB medium agar plate containing ampicillin (100 mg/L) and incubated overnight at 37ºC. Four separate colonies were added to LB medium (6 mL) containing ampicilin (100 mg/L). The culture was incubated overnight (225 rpm, 37°C, 17 h) before dilution in TB (3 mL in 1 L) supplemented with ampicillin (100 mg/L) and riboflavin (1 mg/L). CPR expression was induced with IPTG (0.5 M) at OD_{600} ~ 0.8 (~3 h at 37ºC and 225 rpm). The cultures were further grown at 37ºC for 20 h. Unless mentioned otherwise, all the steps in the protein purification were carried at 4ºC and storage was at -80ºC. Cells were collected by centrifugation (4614 *g*, 25 min) and the harvested cells (46.8 g) were resuspended in TSE buffer (60 mL). Lysozyme

(1.2 mg) was added and the mixture was incubated for 20 min. After centrifugation (3000 *g*, 30 min), the pellet was lysed for 20 min in lysis buffer (60 mL, containing 50 mM Tris base at pH 8.0, 0.5 M EDTA, 10 mg/L aprotinin, and 1 mM PMSF). The cells were further sonicated 7 times at 60% duty cycle and power 8 for 30 s each time with salt-ice bath cooling. Centrifugation (12 000 *g*, 10 min, 4°C) was used to remove the supernatant and the pellet was sonicated again in the same matter. After the second centrifugation, the two batches of supernatant were combined and suspended in affinity buffer (120 mL of: 50 mM Tris base at pH 8.0 containing 10% glycerol (*v*/*v*), 0.1% Triton X100 (*v*/*v*), 0.1 mM EDTA, and 0.05 mM DTT). After another centrifugation (41 000 *g*, 45 min), PMSF (1 mM) was added. A homogenous solution was obtained after 3 h of gentle stirring. The protein solution was applied to a 2',5' ADP Sepharose 4B column (16 mL) equilibrated with affinity buffer (60 mL). The column was first washed with buffer A (40 mL of affinity buffer containing 0.5 mM adenosine), followed by elution of the protein with buffer B (affinity buffer containing 2 mM adenosine 2'-monophosphate (2'-AMP)). The fractions containing the 70 kDa protein were pooled. The sample was loaded onto a fast flow DEAE Sepharose column (8 mL) equilibrated with DEAE-EQ buffer (100 mL of: 0.2 M potassium phosphate buffer at pH 7.4 containing 20% glycerol (*v*/*v*) and 1 g/L CHAPS), and washed with buffer C (16 mL of: DEAE-EQ buffer containing 10 mM ß-mercaptoethanol). The column was washed with buffer C (40 mL) and eluted with a linear gradient increasing from 0.1 to 0.5 M potassium chloride in buffer C. The fractions containing the 70 kDa protein were identified by SDS-PAGE (homogeneous 12.5%) and dialyzed against buffer G (1 L of 0.1 M potassium phosphate buffer at pH 7.4 containing 20% glycerol (*v*/*v*). The overall yield was calculated to be 0.643 mg/L.

CPR concentration and activity assay

The concentration of CPR was determined by oxidation with potassium ferricyanide. An aliquot of CPR (130 µL) was diluted in 100 mM potassium phosphate buffer at pH 7.6 (266 µL) before addition of potassium ferricyanide (7 µM). The concentration was calculated using an extinction coefficient of 21.2 mM^{-1} at 455 nm for the oxidized form of CPR. The activity of CPR was evaluated by cytochrome c reduction in the presence of NADPH. The assay mixture contained CPR (0.5 µM), cytochrome c (50 µM), and NADPH

(100 μM) in 300 mM phosphate buffer at pH 7.6. The blank did not contain NADPH. Cytochrome c reduction by CPR is revealed by absorption peaks at 520 and 550 nm.

AMMC demethylation by CYP2D6

AMMC was dissolved in acetonitrile. For the reference reaction, a mixture (300 μL) containing AMMC (200 μM), CPR (0.7 μM), CYP2D6 (0.12 μM) in potassium phosphate buffer (0.1 M at pH 7.4) was incubated at 37°C for 5 min. The reaction was initiated with the addition of NADPH (0.83 mM) and monitored by fluorescence (l_{ex} = 390 nm, l_{em} = 460 nm) for 15-20 min. Blank reactions were performed in parallel without CYP2D6.

Dextromethorphan demethylation by CYP2D6

Dextromethorphan (DXM) was demethylated to dextrorphan (DXO) by CYP2D6 in the presence of cofactors CPR and NADPH or hydrogen peroxide donors. In the reference reaction, the mixture (300 μL) containing CYP2D6 (200 nM), CPR (825 nM), and DXM (167 μM) in potassium phosphate buffer (0.1 M, pH 7.4) was incubated for 5 min at 37°C. The reaction was initiated with the addition of NADPH (3 mM) and incubated for 1 or 4 h at 250 rpm and 37°C. The reaction was quenched with 23% (*v*/*v*) perchloric acid (100 μL). For the reactions with the hydrogen peroxide donors, CYP2D6 (200 nM) and DXM (167 μM with SPB, SPC, or UHP, and 100 μM with CHP or tBHP) and the mixture did not contain CPR/NADPH. The reactions were initiated by one of different hydrogen peroxide donors (10 mM to 100 mM) or organic peroxides (0.1 mM to 2.5 mM). The reaction was quenched with 23% (*v*/*v*) perchloric acid (100 μL) after incubated for 2 or 4 h at 250 rpm and 37°C. The range of concentrations used for hydrogen peroxide, cumene hydroperoxide and *tert*-butyl hydroperoxide was 0.25 mM to 2.5 mM. The quenched mixtures were centrifuged (16000 *g*, 10 min). The supernatants were filtered through 0.2 μM Teflon filter units before HPLC analysis. Blank reactions without CYP2D6 were carried in parallel.

Quantification of 6β-hydroxytestosterone

Quantification of the 6β-hydroxytestosterone formed during the enzymatic reaction was achieved with a calibration curve obtained by spiking the incubation mixture (no CYP) with 6β-hydroxytestosterone and cortexolone as the internal standard. Concentra-

tions ranging from 0.5 to 5 nmol/mL were used for the calibration curve which was generated by plotting the area ratio (6β-hydroxytestosterone/cortexolone) against the concentration of 6β-hydroxytestosterone. A linear regression plot of peak-area ratio versus concentration was constructed and the concentration of 6β-hydroxytestosterone determined from the peak-area ratio relative to the calibration graph.

Testosterone hydroxylation assays for CYP3A4

The reaction mixtures (300 μL) were prepared by mixing CYP3A4 (580 pmol, 50 μL of a 3.5 μM enzyme solution) and testosterone (115 μM) in potassium phosphate buffer (100 mM, pH 7.4). The reference reaction contained the same components plus CPR (1.5 nmol). The molar ratio of CYP3A4:CPR of 1:4 was selected with regard to near optimal activity as shown by the study of Wang *et. Al.*[3] After 5 min of preincubation at 37°C, the reaction was initiated using various concentrations of SPC, SPB, UHP, CuOOH, *t*BuOOH, or aqueous H_2O_2, and NADPH (1 mM) for the reference reaction. After addition of the solid SPC, SPB or UHP, the reaction mixture was not immediately shaken, to allow the slow release of the hydrogen peroxide as the dissolution occurred. After 15 min the mixture was homogenized by gentle shaking. The reaction was terminated after 1 h of incubation at 37ºC by addition of methylene chloride (500 μL total) immediately followed by introduction of the internal standard cortexolone (15 μL of a 200 μL solution in methanol). The sample was vigorously vortexed, the layers separated by centrifugation (1500 *g* for 2 min), and the organic layer was transferred to a dry vial. The extraction was repeated twice with CH_2Cl_2 (500 μL), and the pooled organic extracts were evaporated to dryness using a roto-evaporator. The residue was redissolved in HPLC-grade methanol (150 μL), vigorously vortexed, sonicated and filtered through a polypropylene syringe filters (0.2 μm pore size, National Scientific Company) before injection on the HPLC. The identity of the products was established by co-elution with authentic standards and by mass spectroscopy. The blank incubation assays were lacking either CYP and NADPH, or the peroxide surrogates, and did not show any detectable products. The extraction efficiency, or recovery, of this procedure was estimated by comparing the peak-area of extracted standards to those of non-extracted standards, and was superior to 96% for both 6β-hydroxytestosterone and testosterone at the concentrations used.

Effect of the buffer in reactions with sodium percarbonate: In order to evaluate the importance of the buffer composition for the reaction with SPC, the experiment was repeated using the conditions described above but in 12 different buffers: 3 potassium phosphate concentrations (0.5 M, 0.75 M and 1 M), and 4 pHs for each (6.0, 6.5, 7.0 and 7.5).

Table S1: Effect of the buffer in CYP3A4 reactions with sodium percarbonate.

Potassium phosphate concentration	pH	% of the activity with CPR/NADPH
500 mM	6.0	86 ± 2.2
	6.5	78 ± 4.3
	7.0	86 ± 2.8
	7.5	15 ± 1.5
750 mM	6.0	61 ± 6.6
	6.5	82 ± 8.1
	7.0	99 ± 4.7
	7.5	63 ± 3.7
1 M	6.0	54 ± 4.1
	6.5	81 ± 5.8
	7.0	**119** ± 3.4
	7.5	77 ± 2.4

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