TECHNICAL ADVANCE

A medium-throughput screening assay to determine catalytic activities of oxygen-consuming enzymes: a new tool for functional characterization of cytochrome P450 and other oxygenases

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Summary

A challenge of the post-genomic era is to determine the functions of a plethora of orphan genes. This is a more acute problem when dealing with large gene families, such as the superfamily encoding cytochrome P450 enzymes in higher plants. We propose here a new, simple, medium-throughput methodology to screen for potential substrates of orphan P450 mono-oxygenases. The same technique can also be applied to screening for inhibitors of the oxygenases involved in the biosynthesis of compounds essential for plant development, such as growth regulators. The method is based on a commercially available microplate system, which detects the oxygen consumed by the catalytic reaction via an oxygen-sensing fluorophore. It is optimized using as a model CYP73A1, the cinnamic acid hydroxylase from *Helianthus tuberosus*, expressed in yeast. We show that the procedure is suitable not only for the detection and real-time monitoring, but also for the quantitative evaluation of enzyme activity. This new method has broad application for the identification of candidate substrates and inhibitors in chemical libraries, to support determination of physiological substrates, development of plant growth regulators, investigations on herbicide and pollutant metabolism, synthesis of valuable compounds and drug design. It also provides a fast-assay platform for determination of catalytic and inhibition parameters. The method applies to plant P450 enzymes, but also to cytochromes P450 from other organisms, and all types of oxygenases. The critical steps, calculation of oxygen consumption from fluorescence signal, and limits of the methods are discussed.

Keywords: oxygenase, screening, oxygen consumption, cytochrome P450, substrate, inhibitor.

Introduction

Cytochrome P450 mono-oxygenases catalyse slow and rate-limiting steps, and also some of the most complex chemical reactions in all plant-specific metabolic pathways, including phenolic and lipid metabolism, biosynthesis of isoprenoids, alkaloids and other amino acid derived compounds (Morant *et al.*, 2003; Schuler and Werck-Reichhart, 2003). Although evolved in plants for adaptive purposes, they also provide valuable precursors for renewable industrial raw materials (e.g. polymers), pharmaceuticals, cosmetics, fragrances and food products. In addition, plant cytochromes P450 are

major players in the detoxification and activation of environmental contaminants, including herbicides, pesticides and other industrial pollutants (Morant *et al.*, 2003). So far, sequencing of plant genomes has revealed 34 full-length cytochrome P450 genes in *Chlamydomonas reinhardtii*, 71 in the moss *Physcomitrella patens*, 246 in *Arabidopsis thaliana*, and 356 in rice (Nelson, 2006). Some P450 genes with essential housekeeping functions are conserved in all species, but many differ significantly from one plant species to another, which reflects the differences in their respective

chemistries. Taking into account the enormous plant chemical diversity, and the average size of the plant genomes, this means that thousands of naturally evolved P450 enzymes will become available in the coming years. These will provide a huge resource for biotechnological applications and production of industrially relevant compounds in plant or microorganism factories, for bioremediation of environmental pollutants, or for optimization of bioactive compounds. The availability of this new resource, however, poses an analytical challenge: the exploration of their catalytic activities and range of potential applications.

According to the most recent update, only 41 of the Arabidopsis P450 genes have been associated with (sometimes scarce and indirect) information concerning their biochemical function(s) (Schuler *et al.*, 2006). The most recent inventory mentioned 83 P450s isolated from all plants functionally characterized using various recombinant systems (*Escherichia coli*, yeast or insect cells; Schuler and Werck-Reichhart, 2003). It is now possible to express large sets of orphan P450 coding sequences in such *in vitro* expression systems, which can provide suitable membrane and redox environments (Barnes, 1996; Lee *et al.*, 1996; Pompon *et al.*, 1996). Much less attainable is the evaluation of their catalytic activities in the absence of sufficient information on the pathways and chemistries to be investigated, while large-scale screening procedures are not available. A few approaches were developed recently to screen for drug metabolism and inhibition of mammalian P450 enzymes in support of drug discovery, or of directed evolution experiments (Ansede and Thakker, 2004; Masimirembwa *et al.*, 2001; Zlokarnik *et al.*, 2005). Some are based on automated and fast, sometimes multiplexed or combinatorial, LC-MS procedures (Ansede and Thakker, 2004; Peng *et al.*, 2003), but procedures based on the detection of radioactive, chromophoric or fluorescent substrates or products are used more extensively for high-throughput approaches. These are used in particular for inhibition-based assays (Crespi *et al.*, 2002; Moody *et al.*, 1999; Stresser *et al.*, 2000; Yamamoto *et al.*, 2002) or molecular breeding (Alcalde *et al.*, 2004; Hannemann *et al.*, 2006; Lussenburg *et al.*, 2005; Nazor and Schwaneberg, 2006). Fluorescent compounds have also been replaced successfully by luminogenic substrates, derived from D-luciferin (Cali *et al.*, 2006).

However, all the above-mentioned screening procedures involved a small number of known and functionalized substrates (fluorescent, luminescent, absorbing at specific wavelength or radiolabelled). No reliable generic test for screening a large sub-set of potential candidate substrates was described. The vast majority of the P450-catalysed reactions imply consumption of one molecule of NADPH (or of electrons from a donor source) and of one molecule of atmospheric oxygen:

We propose here a new method based on the detection of oxygen consumed during P450-catalysed reactions, broadly applicable to any P450 enzyme and a wide array of substrates. This method is a microplate-based assay that uses the Oxygen Biosensor System developed by BD Biosciences (<http://www.bdbiosciences.com>). In each well of the microplate, an oxygen-sensitive fluorophore is embedded in a silicone matrix that forms a layer at the bottom of the well (Wodnicka *et al.*, 2000). The method was initially designed for monitoring the growth rate of microorganisms and mammalian cells, based on their consumption of oxygen (Dike *et al.*, 2006; Guarino *et al.*, 2004; Stitt *et al.*, 2002) and was reported not to be applicable for assays of P450-driven metabolism (Ansede and Thakker, 2004). We describe here the adaptation and optimization of an oxygen-biosensor assay for the screening of plant P450 catalytic activity. The method is optimized using as a model CYP73A1, the cinnamic acid hydroxylase of *Helianthus tuberosus* (Teutsch *et al.*, 1993) expressed in yeast (Pierrel *et al.*, 1994; Urban *et al.*, 1994), and validated using additional yeast-expressed plant cytochromes P450. The assay is fast and simple, and seems broadly applicable. Its first application is the functional characterization of the orphan plant cytochromes P450 revealed by genome analyses. Others include fast determination of catalytic parameters, screening for and design of P450 inhibitors, and screening of potential alternative substrates for industrial applications including biotechnology, biocatalysis and drug design. The method also provides a new approach to the identification of plant P450s playing a significant role in the metabolism of herbicides, pesticides and other environmental pollutants, and in support of the development of new plant protection products.

Results

Detection of a fluorescence change resulting from P450 activity

Figure 1 shows that co-incubation of microsomes isolated from recombinant yeasts expressing CYP73A1 with NADPH and cinnamic acid leads to a fast and linear increase in fluorescence, recorded using the Oxygen Biosensor System. This increase in fluorescence, expected to reflect oxygen consumption in the assay, is very minor in the absence of NADPH or cinnamic acid. A very low consumption of oxygen in the absence of substrate is in agreement with the previously reported tight coupling of the plant cinnamate 4-hydroxylase reaction (Rich and Lamb, 1977; Schalk *et al.*, 1999; Urban *et al.*, 1994). It is also in agreement with negligible expression of endogenous yeast cytochromes P450 under

Figure 1. Evolution of the normalized relative fluorescence (NRF) detected with preparations of microsomes of recombinant yeast expressing CYP73A1: solid line (1), microsomal membranes alone; dotted line (2), microsomal membranes with only NADPH; dashed line (3), microsomal membranes with only cinnamic acid; dots/dashes (4), microsomal membranes with NADPH and cinnamic acid.

The assay contained 16.7 pmol recombinant cytochrome P450 (CYP73A1), 0.1 mM NADPH, 3 mM glucose-6-phosphate, 0.4 U of glucose-6-phosphate dehydrogenase and 0.2 mM cinnamic acid in buffer A, and was run at 27°C. Excitation wavelength was 486 nm and emission fluorescence was monitored at 620 nm.

the growth conditions used for production of the recombinant enzymes. However, a clean, clear-cut signal was obtained only after optimization, as shown below.

The initial technique was designed to monitor the growth rate of microorganisms or of animal cell cultures (Dike *et al.*, 2006; Guarino *et al.*, 2004; Wodnicka *et al.*, 2000). It assumed, in a first approximation, that the microorganisms or thin cell layer did not interfere with the rate of oxygen diffusion (Guarino *et al.*, 2004). In the same way, we can also assume that a suspension of microsomal membranes does not create a barrier for oxygen diffusion. However, the suspension of membrane vesicles is not a homogeneous solution, and particles in suspension tend to sediment during the time-course of the reaction. To minimize sedimentation of the microsomal particles, 20% glycerol was added to the assay.

A more consistent problem was due to temperature equilibration. Dissolved oxygen concentrations vary depending on environmental conditions, in particular as a function of temperature. When the reaction is initiated, all the components (well, silicon matrix, reaction buffer) must be at the same temperature. The reaction temperature is 27°C. Adding reaction buffer at 20°C to a plate pre-incubated at 27°C leads to a long readjustment of fluorescence, due to the readjustment of oxygen concentrations and equilibration with the sensor (Figure 2a). It prevents correct measurement of initial enzyme activity (Figure 2b). Pre-incubation of the reaction medium without microsomes in the fluorimeter to warm up the solution at 27°C before addition of microsomes, also preheated for 2 min at 27°C, avoids

Figure 2. Impact of temperature equilibration on dissolved oxygen and fluorescence changes detected in the assay.

(a) Evolution of normalized relative fluorescence resulting from temperature equilibration in buffer A.

(b) Evolution of normalized relative fluorescence in the assay without pre-equilibration of the reaction buffer at 27°C. Concentration of reactants as in Figure 1. Dotted line (1), microsomal membranes alone; dashed line (2), microsomal membranes with only NADPH; solid line (3), microsomal membranes with only cinnamic acid; dots/dashes (4), microsomal membranes with NADPH and cinnamic acid.

significant fluorescence readjustments and leads to reliable measurement of initial activities (Figure 1).

Enzyme is the less stable reactant in the assay

One of the advantages of the assay is real-time monitoring of the reaction, which provides time-courses of activity (which are unattainable or overly time-consuming in a standard radiometric or HPLC assay). As shown in Figure 1, such monitoring reveals that the reaction is linear for only 40–50 min, then slows down and stops. This is followed by a re-equilibration of atmospheric oxygen with the medium. In order to determine the reason for the arrested reaction, the initial concentrations of NADPH, cinnamic acid and microsomes were restored in the assay

Figure 3. Restoration of activity in the assay after arrest. Changes in the normalized relative fluorescence observed on addition of 0.1 mM NADPH (dashed line); 0.2 mM cinnamic acid (dotted line); or 16.7 pmol CYP73A1 (dots/dashes) in the assay at 120 min incubation (Figure 1).

after complete reaction arrest. Figure 3 shows that only addition of microsome membranes restores the initial activity. The reaction arrest observed after 40 min is thus due not to substrate or NADPH depletion, but to the inactivation of the P450 enzyme.

A short adjustment phase is often observed in the first 2 min of the assay. Ideally, the initial enzyme activity has to be determined in a window between 5 and 20 min incubation. The short lag phase observed at the beginning of the reaction usually depends on the substrate and P450 investigated, and in some cases may be due to slower equilibration of hydrophobic substrates with the membranes. In the same way, the inactivation rate at 27°C depends on each P450 enzyme. Optimization of a time window is therefore needed, not for screening purposes, but for accurate and quantitative measurements of enzyme activity.

The signal increases linearly with enzyme and NADPH concentrations

One of the main limits of the assay for substrate screening purposes is the ratio of the signal observed in the presence of a candidate substrate to the signal obtained when NADPH alone is added to the suspension of microsomes. Background oxygen consumption can be limited by optimizing the concentrations of microsomes and NADPH in the assay. In an attempt to optimize such parameters, we compared oxygen consumption recorded with different concentrations of microsomal membranes and different concentrations of NADPH in the assay. Figure 4(a) shows that the signal increases linearly with enzyme concentrations ranging from 3 to 30 pmol per assay. Low signal due to yeast membrane constituents can be deduced, running a parallel experiment in the absence of substrate and/or NADPH.

Figure 4. Changes in normalized relative fluorescence resulting from increased concentrations in CYP73A1 (panel A) and NADPH (panel B) in the assay.

(a) (▼) Microsomal membranes along, (■) microsomal membranes with only NADPH, (●) microsomal membranes with only cinnamic acid, (▲) microsomal membranes with NADPH and with cinnamic acid.

(b) (▲) microsomal preparation with only NADPH and (●) microsomal preparation with NADPH and with cinnamic acid.

Initial rates of increase of NRF at 27°C were determined with CYP73A1 varying from 3.34 to 33.4 pmoles in the assay. For clarity of the figure, the linear regression was drawn only for the assay containing microsomes, cinnamic acid and NADPH. Data are means of three independent measurements.

The signal also increases linearly with NADPH concentration, with saturation around 100 μM (Figure 4b). At a 100 μM concentration the background signal remains very low. This NADPH concentration was thus used for further measurements. NADPH concentration may, however, be adapted for each enzyme and recombinant system for optimal results.

Oxygen consumed by the cytochrome P450-catalysed reaction

Application of the Stern–Volmer equation after experimental determination of the thermodynamic range constant DR (Eqn. 1 in Experimental procedures) allows us to convert the fluorescence signal to apparent oxygen consumption rates (OCR) using Eqn. 3. The calculation assumes that the

reaction is carried out under homogeneous conditions with no significant formation of O_2 gradient within the reaction volume, and that the oxygen equilibration rates with the atmosphere are negligible within the initial 30-min time frame of the experiment. Addition of mineral oil on top of the reaction medium did not significantly improve the signal, which validates the latter assumption. Linear responses to enzyme and NADPH concentration provide an empirical demonstration of the linear response of the sensor to oxygen concentration in the assay, despite its delayed equilibration with the assay compartment.

Application to determination of catalytic activity and kinetic parameters of a reaction

In a P450-catalysed reaction, the number of oxygen molecules consumed theoretically equals the number of substrate molecules converted. In order to confirm further the reliability of the method, and to test whether it provides a correct estimate of enzyme activity, we first compared activity measured by conventional HPLC detection of substrate and product with oxygen-sensor assays. Figure 5(a) shows that initial velocities evaluated using the two assays are very comparable, but that enzyme inactivation appears slower in the oxygen-sensor assay. Faster inactivation possibly results from higher dilution or higher oxygenation (agitation) of the enzyme in the HPLC assay.

In a second step, we attempted to determine the catalytic parameters of the 4-hydroxylation of cinnamic acid by CYP73A1, and of the 3'-hydroxylation *p*-coumaroyl shikimate by CYP98A3 using the oxygen-sensor assay. Figure 5(b) shows, in the case of CYP73A1, that the method provides a good quality kinetic analysis. Resulting computed catalytic parameters were K_M of $17 \pm 2 \mu M$, K_{cat} of $546 \pm 30 \text{ min}^{-1}$, and k_{cat}/K_M of $32 \pm 6 \text{ min}^{-1} \mu M^{-1}$ in the case of CYP73A1; K_M of $5 \pm 1 \mu M$, K_{cat} of $1380 \pm 60 \text{ min}^{-1}$, and k_{cat}/K_M of $276 \pm 12 \text{ min}^{-1} \mu M^{-1}$ for CYP98A3. Such values are very similar to those reported previously for CYP73A1, CYP98A3 and related enzymes by more conventional methods (Ehltling *et al.*, 2006; Morant *et al.*, 2007; Schalk *et al.*, 1997; Schoch *et al.*, 2001; Urban *et al.*, 1994). Compared with one to two working day for a classical TLC- or HPLC-monitored experiment, the whole analysis (calculations included) can be performed in <2 h.

Favourable comparison with HPLC determination of initial enzyme velocity and catalytic parameters thus validates the method for evaluation of enzyme-dependent OCRs and deduced catalytic activities.

Application to screening for potential substrates and ligands

To confirm the applicability of the method as a screening procedure, a collection of molecules selected as representative of a large spectrum of chemical structures (list

Figure 5. Evaluation of CYP73A1 enzymatic activity by the oxygen-sensor assay.

(a) Comparison of cinnamate 4-hydroxylase activities determined by HPLC (▲) and oxygen sensor (●) assays. The fluorimetric assay contained 0.2 pmol of recombinant cytochrome P450 (CYP73A1), 0.1 mM NADPH, 3 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase and 0.2 mM cinnamic acid in buffer A, and was run at 27°C. In the HPLC assay, 4-hydroxycinnamic acid was quantified at 308 nm.

(b) Fluorimetric determination of the kinetic parameters of CYP73A1 for cinnamic acid. Initial rates of activity were deduced from oxygen consumption computed from the measured NRF values. Data are means of three independent experiments.

provided in Supplementary Material) was screened for metabolism by CYP73A1, and CYP76C4, CYP76G1, CYP72A6, CYP76C2 and CYP7A8, a small sub-set of still-orphan enzymes of our recombinant collection. Cinnamic acid was included in the collection of molecules tested as a positive control for CYP73A1.

In addition to cinnamic acid, a single molecule provided a significant positive signal (compound H10 in the list: benzyloxygramine) with CYP73A1. This molecule shares with cinnamic acid an unsubstituted benzylic ring structure (Figure 6), potentially substrate of the enzyme. It was tested for metabolism in an HPLC assay, which did not confirm metabolism of the compound into a more polar metabolite. Benzyloxygramine was thus considered as a potential ligand

Figure 6. Natural substrate (a, cinnamic acid) and additional ligand (b, benzyloxygramine) of CYP73A1 identified in preliminary screening experiments.

of CYP73A1, inducing abortive oxygen consumption. Tested as an inhibitor of cinnamate 4-hydroxylase activity in an HPLC assay, it was confirmed to behave as an inhibitor: 1.2 mM (0.33 mg ml⁻¹) benzyloxygramine reduced cinnamate 4-hydroxylase (CAH) activity by 75% when measured with 20 μ M cinnamic acid in the assay (data not shown).

In the case of CYP72A8, a single compound in the collection of molecules provided a clear positive signal, indicating oxygen consumption. This compound is the pharmacologically active alkaloid quinidine (Figure 7a), with anti-arrhythmic and anti-fibrillant properties, which was previously shown to behave alternatively as a substrate or an inhibitor of some drug-metabolizing mammalian P450 enzymes (Bhoopathy *et al.*, 2002; McLaughlin *et al.*, 2005). In

Figure 7. Quinidine metabolism by CYP72A8.

(a) The quinidine substrate of CYP72A8.
 (b) MALDI-TOF-MS mass spectra obtained in positive reflectron mode representing the mono-isotopic [M + H]⁺ masses of the substrate (S) = 324.998 m/z and the corresponding oxidized + 16-Da product (P) = 340.987 m/z. Microsomes from yeast expressing CYP72A8 (12 pmol in the assay) were incubated for 2 h at 27°C, with 3 mM quinidine, 3 mM glucose-6-phosphate, 0.4 U of glucose-6-phosphate dehydrogenase, with or without 100 μ M NADPH, and in a total volume of 300 μ l 20 mM NaPi, pH 7.4. The oxygenated product (P) is detected only in the assay incubated in the presence of NADPH, but not with microsomal fraction of recombinant yeast alone.

order to confirm its oxygenation, the reaction product was analysed by mass spectrometry. This analysis clearly indicates the formation of an *M* + 16 product in the assay, only when carried out in the presence of NADPH (Figure 7b). The position of quinidine oxygenation was not determined. Quinidine is a natural compound, and the D-isomer of the anti-malarial drug quinine. It is extracted together with quinine and cinchonin from the bark of the tropical cinchona tree. Quinine, quinidine and their precursors cinchonin and cinchonidin are typically associated in classical anti-malarial treatments. However, production of quinine-like alkaloids has not been reported so far in *A. thaliana*.

Application to screening for potential inhibitors

The availability of a fast and efficient procedure to screen for potential P450 enzyme inhibitors would be a valuable support to the development of active compounds in human health, pest and plant growth control research. The oxygen-consumption assay was thus tested for direct detection of

Figure 8. Inhibition of CYP73A1 detected via the oxygen-sensing assay.

(a) Inhibition of CYP73A1 by increasing concentrations of 2-hydroxy-1-naphthoic acid (competitive inhibitor).
 (b) Determination of the *K_i* of 2-hydroxy-1-naphthoic acid for CYP73A1. The assays contained 16.7 pmol recombinant CYP73A1, 0.1 mM NADPH, 3 mM glucose-6-phosphate, 0.4 U of glucose-6-phosphate dehydrogenase and 0.2 mM cinnamic acid in buffer A. They were run at 27°C with concentrations of 2-hydroxy-1-naphthoic acid ranging from 0.05 to 5 μ M. Data were averaged over four independent measurements.

P450 inhibition. Figure 8 shows inhibition of cinnamate 4-hydroxylase activity of CYP73A1 by 2-hydroxy-1-naphthoic acid, previously described as a competitive inhibitor of the enzyme (Schalk *et al.*, 1997). As expected, inhibition of oxygen consumption increases with increased inhibitor concentration (Figure 8a). Figure 8(b) shows that the method could be applied with success for the determination of the K_i of 2-hydroxy-1-naphthoic acid for CYP73A1. The value determined with the oxygen-sensing method was 56 ± 8 versus 48 nM by a standard TLC determination of cinnamate substrate conversion (Schalk *et al.*, 1997).

In order to confirm the applicability of the method as a screening procedure for the characterization of new inhibitor, the above-mentioned collection of 80 molecules representative of a diversity of structures (Supplementary Material) was also screened for inhibition of CYP73A1. In this case, 2-hydroxy-1-naphthoic acid and methyl-2-naphthoic acid, both competitive inhibitors of CYP73A1 (Schalk *et al.*, 1997) with previously reported K_i of 48 and 98 μ M, respectively, were included in the collection as positive controls. These two compounds only were detected as inhibitors in the screening experiment. The oxygen-sensing method thus appears highly suitable for the detection and evaluation of P450 enzyme inhibition.

Discussion

We show here that the monitoring of oxygen consumption in a microplate assay offers a powerful approach for the detection of cytochrome P450-catalysed reactions in membrane fractions of recombinant organisms. The method can be used to screen for potential substrates, but also for fast evaluation of the catalytic parameters. In addition to substrates, the method also detects potential inhibitors very efficiently. The major limitations of the method are due to background oxygen consumption. Background consumption can be negligible, for example in the case of yeast-expressed CYP73A1, or may become significant depending on: (i) the level of expression of cytochrome P450 in a given recombinant organism; and (ii) the uncoupling of the reaction (consumption of oxygen in the absence of any added substrate), which might be an intrinsic property of a given P450 or due to the presence of a substrate such as a fatty acid or a sterol in the microsomes of the recombinant organism. It must also be kept in mind that uncoupling can be induced by the binding of any ligand, such as a poor substrate or inhibitor that displaces the sixth ligand (usually H_2O) present in the resting active site. The method is thus highly suitable for screening for natural substrates, fast-metabolized exogenous compounds and inhibitors. It is, however, not suitable for detection of very low metabolism of drugs or long drug stability assays.

The method offers broad potential applications. The first is the screening of libraries of natural or synthetic compounds

for metabolism, in support of the functional characterization of orphan P450 enzymes. A collection of over 100 recombinant plant cytochromes P450 expressed in yeast, *E. coli* or insect cells has been constructed in our laboratory. More than 80% of these have catalytic functions that are unknown or only partially explored. If all their potential substrates are not available, vast libraries of natural and synthetic compounds have been organized in recent years for screening of therapeutic targets, and such libraries can provide leads to natural functions of the orphan P450s. The second most obvious application of this screening procedure is characterization of the plant P450s most active in herbicide, insecticide and fungicide metabolism. Once a sub-set of relevant enzymes can be identified, the same procedure can be applied, at least to preliminary screenings for lead optimization for metabolism and interaction with other active material. Last, but not least, the technique seems very promising for the discovery of new plant cytochrome P450 inhibitors. As one to several P450 oxygenases seem to be involved in the biosynthesis or catabolism of most plant hormones and signalling molecules, this may pave the way to the discovery of new plant growth regulators.

In addition to such plant- and crop-focused applications, extensive investigation of plant P450 catalytic potential is also expected to reveal new tracks for the industrial application of available natural catalysts to the synthesis of renewable materials, antioxidants, and other bioactive compounds. Among other potential applications of the collections of available plant enzymes are the optimization of new drugs for potency, selectivity, physicochemical properties, toxicity and ADME (absorption, distribution, metabolism and excretion). The technique is also applicable to non-plant P450s such as drug-metabolizing P450s, and all other oxygenases.

Experimental procedures

Chemicals

Cinnamic acid, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 2-hydroxy-1-naphthoic acid and methyl-2-naphthoate were purchased from Sigma-Aldrich (<http://www.sigmaaldrich.com>). Oxygen Biosensor plates were from BD Biosciences (<http://www.bdbiosciences.com>). A collection of 80 molecules representative of a diversity of chemical structures was selected in the chemical library of the Faculty of Pharmacy (partner of the National Chemical Library) of the University Louis Pasteur of Strasbourg, and kindly provided by Drs Jean-Jacques Bourguignon and Bruno Didier.

Yeast production of CYP73A1 and CYP98A3 and preparation of microsomal membranes

The *Saccharomyces cerevisiae* strain WAT11, expressing the cytochrome P450 reductase ATR1 from *Arabidopsis thaliana* under control of the galactose-inducible promoter *GAL10-CYC1*, transformed with the vector pYeDP60 containing the coding sequence of CYP73A1 from *Helianthus tuberosus* or CYP98A3 from *A. thaliana*

under the control of the same promoter, was used for the production of recombinant proteins. Yeasts were grown, protein expression induced and microsomal membranes prepared as described previously (Schoch *et al.*, 2001; Urban *et al.*, 1994). Expression of P450 proteins was estimated from the CO-bound-reduced versus reduced difference UV-vis spectra of microsomal preparations, using the extinction coefficient determined by Omura and Sato (1964) of $91 \text{ M}^{-1} \text{ cm}^{-1}$. Microsomes were stored at -30°C in Tris 50 mM, EDTA 2 mM, pH 7.5 containing 30% glycerol.

Fluorimetric assay for enzyme activity

The assay uses standard BD Oxygen Biosensor System 96-well plates with an oxygen-sensitive fluorophore, tris(4,7-diphenyl-1,10-phenanthroline) ruthenium (II) chloride, incorporated in a permeable silicone rubber matrix at the bottom of each well. The matrix permeability allows oxygen exchange with the solution in the well. The volume of each well is around 300 μL , and the volume of the fluorophore matrix lining the bottom, 7.6 μL .

Oxygen consumption due to substrate oxygenation was measured at 27°C on an SX18MV-R fluorimeter apparatus (Bio-Tek, <http://www.biotek.com>). The excitation wavelength was set at 486 nm and the emitted light was collected at 620 nm. All experiments were carried out in sodium phosphate buffer, 20 mM, pH 7.4 containing 20% glycerol (buffer A) in order to avoid sedimentation of the microsomal particles at the bottom of the well during the reaction. The NADPH concentration in standard experiments was 0.1 mM, and microsome concentration ranged from 3 to 33 pmol P450. Glucose-6-phosphate, 3 mM, and glucose-6-phosphate dehydrogenase, 0.4 U per well, were added to maintain saturating NADPH concentration and prevent inhibition of the P450 reductase by oxidized NADPH. The reaction medium was preheated at 27°C in the fluorimeter in order to equilibrate oxygen concentration between the liquid phase and the solid phase containing the embedded fluorophore, before initiation of the reaction by adding microsomes also pre-equilibrated at 27°C . Fluorescence was recorded immediately after addition of the microsomes for normalization. Fluorescence was then recorded every 30 sec for 2 h.

For saturation kinetics, NADPH and cinnamic acid were varied from 0.5 to 500 μM and 3 to 200 μM , respectively. In inhibition kinetics experiments, inhibitor concentrations varied from 0.02 to 2 μM . All the screening procedures were carried out using final concentrations of candidate substrate or inhibitors of 0.33 mg mL^{-1} in the assays. Screening for new inhibitors was performed using a cinnamic acid final concentration of 20 μM .

All experiments have been repeated several times, and provided very similar results. A representative experiment is shown for real-time changes in fluorescence. For quantitative evaluations, data are means of three to four experiments. Standard deviations (not shown) were always <10% of the calculated average values.

Although meant for single use, we have recycled plates for several measurements without significant impact on the results. However, this means handling with care and allowing re-equilibration with atmospheric oxygen.

Calculation of oxygen consumption

Fluorescence changes in the assay have to be converted into variations in dissolved oxygen concentration in order to evaluate substrate metabolism (assuming substrate metabolism is the only source of oxygen consumption). As described by Guarino *et al.* (2004), fluorescence of tris (1,7-diphenyl-1,10 phenanthroline) ruthenium (II) chloride in a static system at equilibrium, but also in

transient pre-equilibration phase (Dike *et al.*, 2006), is directly proportional to the oxygen concentration in the medium and follows the Stern–Volmer equation:

$$\frac{I_0}{I} = 1 + K_{SV} \times [\text{O}_2] \quad (1)$$

where I_0 is the fluorescence intensity in the absence of oxygen, I is the fluorescence intensity at a defined oxygen concentration, K_{SV} is the Stern–Volmer constant and $[\text{O}_2]$ the oxygen concentration (in atm). To convert the fluorescence signal into dissolved oxygen concentration, it is necessary to know the value of the Stern–Volmer constant. This constant varies with the plate reader and can be calculated after measuring the dynamic range constant $\text{DR} = I_0/I_A$. I_A is the fluorescence intensity at ambient oxygen concentration. The DR can be calculated after adding sodium disulphite to remove all the oxygen in the solution to form Na_2SO_4 .

In practice, 250 μL of 100 mM sodium disulphite in PBS buffer is incubated in a well of the microplate. After 1 h, all the oxygen in the solution and sensor is consumed, and the maximum fluorescence emission intensity at I_0 is then used to calculate DR and the value of (K_{SV}) from:

$$K_{SV} = \frac{\text{DR} - 1}{[\text{O}_{2a}]} \quad (2)$$

where $[\text{O}_{2a}]$ = ambient oxygen concentration = 0.209 atm. Oxygen concentrations can then be deduced from fluorescence intensities using the equation:

$$[\text{O}_2] = \left\{ \frac{\text{DR}}{(I/I_A)} - 1 \right\} / K_{SV} \quad (3)$$

Apparent OCR values expressed in $\mu\text{mol min}^{-1} \mu\text{mol}^{-1}$ P450 were computed from the slope of the linear segment of the oxygen-consumption curves recorded for each assay.

Enzyme activity measurement by HPLC analysis

Enzyme activity with CYP73A1 was measured by following the rate of formation of the metabolite 4-hydroxy cinnamic acid. Assays contained 0.1 mM NADPH, 3 mM glucose-6-phosphate, 0.4 U of glucose-6-phosphate dehydrogenase, 0.4 pmol P450 as microsomal membranes and 200 μM cinnamic acid in sodium phosphate buffer, 20 mM, 20% glycerol, pH 7.4 in a final volume of 100 μL . The reaction was carried out at 27°C , initiated by adding microsomes and terminated by addition of 10 μL HCl 4 M. The products were extracted twice with 500 μL ethyl acetate, the organic phase was pooled and evaporated under argon, and the residue, dissolved in 160 μL 10% acetonitrile, 90% water, 0.2% acetic acid (v/v/v), was analysed by reverse-phase HPLC [LiChrosorb RP-18 (Merck, <http://www.merck.com>), $4 \times 125 \text{ mm}$, 5 μm ; flow rate 1 mL min^{-1} ; 5 min isocratic 10% acetonitrile, then 20-min linear gradient from 10 to 52% acetonitrile in water containing 0.2% acetic acid]. Absorbance of the eluant was monitored at 308 nm with a diode-array detector. Quantification of the product was deduced from the 4-hydrocinnamate peak surface, with reference to a calibration curve resulting from extraction and analysis of known amounts of *p*-coumaric acid added to blank assays. Incubations for inhibition experiments were carried out in the presence of 20 μM cinnamic acid at 27°C for 5 min. The products were extracted and quantified as above.

MALDI-TOF-MS analysis of reaction product

Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on an Ultraflex TOF-TOF mass spectrometer (Bruker Daltonics, <http://www.bdal.de>).

The instrument was used at a maximum accelerating potential of 25 kV and was operated in reflector-positive mode. Ionization was accomplished with a 337-nm beam from a nitrogen laser with a repetition rate of 20 Hz. The output signal from the detector was digitized at a sampling rate of 2 GHz. For MS, the samples were treated as follows: samples were prepared by standard dried droplet preparation on stainless steel MALDI target plates (Bruker Daltonics) using 2,5-dihydroxybenzoic acid (DHB). The DHB matrix used was a freshly saturated solution in acetonitrile and water 50:50 (v:v). A 0.5 µl aliquot of sample reconstituted in a solution of acetonitrile and water 50:50 (v:v) was spotted on the target, followed by 0.5 µl saturated matrix solution. External calibration of MALDI mass spectra was carried out using the singly charged mono-isotopic peak of six peptides, Bradykinin (757.399), angiotensin II (1046.582 Da), substance P (1347.735 Da), bombesin (1619.822 Da), Renin (1758.933), ATCH (2465.198 Da) (Sigma-Aldrich). Mono-isotopic peptide masses were automatically annotated using FLEXANALYSIS 2.0 (Bruker Daltonics).

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Supplementary materials

The following supplementary material is available for this article online:

Figure S1. List and structure of the compounds used in the screening for substrates and inhibitors.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

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