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A computational protocol to predict suitable redox mediators for substitution of NAD(P)H in P450 monooxygenases

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ABSTRACT

P450s catalyze a wide spectrum of stereo- and regioselective reactions like hydroxylations, epoxidations and dehydrogenations. Therefore biotransformations with P450s are of great relevance to organic synthesis. The use of isolated enzymes offers advantages over the use of whole cells. A key issue for catalytic applications of isolated P450s is the demand for a continuous electron supply to the heme-group. Mediator driven bioelectrocatalysis can help to overcome this problem.

For mediator driven bioelectrocatalysis the identification of a suitable mediator is crucial for the fast development of an efficient electro enzymatic process. To this end we have developed a computational screening method based on using freely available software. Calculated electron transfer rates were compared with measured product formation rates. The novel *in silico* procedure allows a faster identification of suitable mediators for electrochemically driven P450 catalyzed reactions and can be used as screening tool. It may also lead to a massive reduction of experimental effort for the development of bioelectrochemical reaction systems in the future.

1. Introduction

Cytochrome P450 monooxygenases (P450s) constitute a large family of heme enzymes present in the genomes of all biological kingdoms [1]. P450s catalyse chemical "dream reactions" such as regio- and stereospecific hydroxylations of non-activated carbon-hydrogen bonds by inserting one oxygen atom from molecular oxygen into the substrate. For this reaction the enzyme requires the cofactor NAD(P)H as "electron" donor. The overall reaction is given in the following equation:

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + NAD(P)^+ + H_2O$$

P450s catalyse further reactions, *e.g.* epoxidations of double and triple bonds, heteroatom oxidations and dealkylation [2].

Their catalytic versatility makes P450s attractive for organic synthesis in chemical and pharmaceutical industries [3]. Although the range of reactions catalyzed by P450s is diverse, a key issue for catalytic applications of isolated P450s is the demand for a continuous electron supply to the catalytic heme centre. *In vivo*, reduction equivalents are supplied by cellular NAD(P)H which is, however, far too expensive to be used in equimolar concentrations in technical applications [4]. Therefore, several enzymatic [5–7], photochemical [8,9] or electrochemical [10–13] approaches substituting or recycling NAD(P)H during biocatalytic reactions have been explored but most of them still suffer from limited efficiency [14]. A significant number of investigations carried out in recent years have focused on replacing cofactors by electrochemically regenerated mediators, *i.e.* redox active molecules that transfer the electrons from the electrode to the P450s [15–19]. P450cin, isolated from *Citrobacter braakii*, catalyzes the stereoselective hydroxylation of 1,8-cineole [20]. The hydroxylation is the first step in a metabolic pathway of *Citrobacter braakii* to utilize cineole as its sole source of carbon and energy. The P450cin system is a three component system; the heme containing protein (CinA) hydroxylates cineole, a FAD containing protein (CinB) accepts the "electrons" from NADPH, and a FMN containing protein (CinC) transfers electrons from CinB to CinA. Recently we have shown that phenosafranine and safranine T enable electron transfer in absence of NADPH, cindoxin, and cindoxin reductase, thereby illustrating that none of the natural redox partners is required for product formation [21]. The experimental identification of a suitable mediator-enzyme combination starts with the measurement of the formal potential of the active site of CinA. The formal potential is defined as the average of the cathodic and anodic peak potentials ($E^{\circ\prime} = (E_{pc} + E_{pa})/2$).

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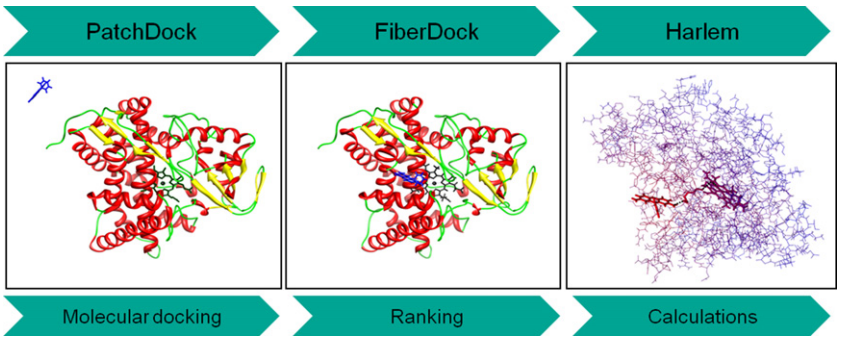

Fig. 1. Scheme of the docking with CinA and phenosafranine; PatchDock - Molecular docking algorithm based on shape complementarity (http://bioinfo3d.cs.tau.ac.il/PatchDock/); FiberDock - Refinement of molecular docking results by restricted interface side-chain rearrangement, by backbone minimization in the directions of calculated normal modes and by soft rigid-body optimization (http://bioinfo3d.cs.tau.ac.il/FiberDock/); HARLEM - calculates the electron transfer pathways and corresponding rates in proteins (http://www.kurnikov.org/harlem_manual/html/index.html). The structures shown always include the mediator molecule (here phenosafranine). In the scheme constructed by HARLEM the colour gradient (red to blue) illustrates the predicted electron flow pathway between the mediator (red) and the heme centre (purple).

To perform a reduction of the active site the redox potential of the mediators must be more negative than the measured potential at the active site of CinA. The following step of the investigation typically includes testing of different mediators in combination with the chosen enzyme and measurement of the corresponding product formation rates. However, this approach is experimentally time-consuming and identification of suitable mediators depends on experience and "luck". Therefore we have investigated the binding of different mediators to P450cin with docking tools for an *in silico* prediction of suitable mediators for the substitution of NADPH and the natural redox partners CinB and CinC. A protein-mediator docking algorithm consists of two essential components, sampling and scoring. Sampling refers to the generation of potential mediator binding conformations. Scoring is the prediction of the binding tightness for individual conformations with a physical or empirical energy function. The best conformation, namely the one with the lowest energy score, is predicted as the binding mode. The last step of our prediction method is the calculation of the electron transfer rate. Figure 1 shows our docking procedure. As an example the docking of phenosafranine as mediator to P450cin is shown.

A simple empirical expression for electron transfer rate is given by equation [22,23]:

$$\log k_{ET} = 15 - 0.6R - 3.1\frac{\left(\Delta G^0 + \lambda\right)^2}{\lambda}$$

The equation expresses an exponential dependence of electron transfer rate on edge-to-edge distance (R, in Å) and a parabolic dependence of log rate on Gaussian free energy (ΔG°, in eV) and reorganisation energy (λ, in eV) [23].

2. Experimental

2.1. Reagents and materials

All chemicals were analytical grade or of higher quality and purchased from Fluka (Buchs, Switzerland) or Sigma-Aldrich (Taufkirchen, Germany). 2α-Hydroxy-1,8-cineole was synthesized according to Miyazawa *et al.* [24]. *Escherichia coli* BL21(DE3) chemically competent cells were obtained from Novagen (Darmstadt, Germany). P450cin heme domain (CinA) gene was purchased from Geneart AG (Regensburg, Germany).

2.2. Cultivation of E. coli and expression of P450cin

A single colony of *E. coli* BL21(DE3) cells containing pET-28a+ plasmid with CinA was picked from overnight plates and used to inoculate 5 ml Luria broth (LB) medium supplemented with 30 μg kanamycin/ml. The overnight cultures were used to inoculate 50 ml terrific broth (TB) medium containing 30 μg kanamycin/ml in 300 ml flasks. One litre of the TB medium contained 12 g tryptone, 24 g yeast extract, 4 ml glycerol, 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4. The salts were added after autoclaving. Cells were grown at 37 °C and 180 rpm until the OD600 reached 0.8. Then the expressions were induced by adding 1 mM β-D-thiogalactopyranoside (IPTG) and 0.9 mM 5-aminolevulinic acid hydrochloride (ALA). Cells were grown for a further 18 h at 25 °C and 180 rpm. Cells were harvested by centrifugation at 10,000 g for 20 min at 4 °C and resuspended in 25 mM potassium phosphate buffer (pH 7.5). Cells were disrupted by sonification on ice (3 times, 90 s, amplitude 35%, Branson sonifier, Dietzenbach, Germany). Cell debris were removed by centrifugation at 20,000 g for 20 min at 4 °C. The cell free extract was filtered through a 0.45 μm filter unit. CinA concentrations were estimated by CO difference spectroscopy [25].

2.3. Electrochemical reaction system

Experiments were performed under potentiostatic control (Reference 3000 Potentiostat/Galvanostat/ZRA, Gamry instruments, USA) operating a three electrode system consisting of a Pt (surface area A = 2 cm^2) working electrode, an Ag/AgCl (sat. KCl) reference electrode and a Pt counter electrode. The reactions (total volume 1 ml) were performed in 50 mM potassium phosphate buffer (pH 7.5) at room temperature with 1 μM CinA, 6 mM 1,8-cineole and 1500 U/ml catalase. Mediator solutions of 5 mM cobaltsepulchrate (CoSep), 1.2 mM phenosafranine (PSF), 1.2 mM safranine T (SAF), 0.6 mM FAD, 0.6 mM FMN, 0.8 mM methyl viologen (MV), 0.8 mM ethyl viologen (EV), 1.2 mM neutral red (NR), 1.2 mM riboflavin (RF), 1.2 mM Janus green B (JGB) were supplemented individually to the reaction mixture and to start the reaction, working electrode was polarized to -750 mV. One ml reaction mixture was extracted to ethyl acetate (200 μl for NR, RF and JGB; 250 μl for SAF, PSF, FAD and FMN; 1 ml for CoSep) and dried with Na_2SO_4 and then analyzed by GC-MS (GC17A with Q5050 mass spectrometer, Shimadzu, Duisburg, Germany) as follows: column, VB-5 (Valcobond, 30 m × 0.25 mm × 0.25 μm); temperature program, from 80 °C, with 6 °C min^{-1} to 160 °C, hold for 2 min. Mass spectrum data of the 2β-hydroxy-1,8-cineole is listed in form of the major fragment ions with their relative intensities in parentheses. m/z: 170 (M+), 155 (tr), 137 (1), 126 (33), 111 (21), 108 (50), 93 (27), 83 (19), 71 (50), 69 (30), 55 (22), 43 (100) [21].