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Understanding of real alternative redox partner of *Streptomyces peucetius* DoxA: Prediction and validation using *in silico* and *in vitro* analyses

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ABSTRACT

Streptomyces peucetius ATCC27952 contains the cytochrome P450 monoxygenase DoxA that is responsible for the hydroxylation of daunorubicin into doxorubicin. Although *S. peucetius* ATCC27952 contains several potential redox partners, the most suitable endogenous electron-transport system is still unclear; therefore, we conducted a study of potential redox partners using Accelrys Discovery Studio 3.5. Recombinant DoxA along with its redox partners from *S. peucetius* FDX1, FDR2, and FDX3, and the putidaredoxin and putidaredoxin reductase from *Pseudomonas putida* that are essential equivalents of the class I type of bacterial electron-transport system were over-expressed and purified. The successful development of an efficient redox system was achieved by an *in vitro* enzymatic catalysis reaction with DoxA. The optimal pH for the activation of the heme was 7.6 and the optimal temperature was 30 °C. Our findings suggest a two-fold increase of DoxA activity via the NADH → FDR2 → FDX1 → DoxA pathway for the hydroxylation of the daunorubicin, and indicate that the usage of a native redox partner may increase daunorubicin-derived doxorubicin production due to the inclusion of DoxA.

1. Introduction

It is acknowledged that the cytochrome P450 (CYP) oxygenases that can catalyze a diverse set of often highly specific oxidation reactions have a high biocatalytic potential in the chemical and pharmaceutical industries [1], as they are involved in the biosynthesis of several pharmaceuticals such as polyketide antibiotics, artemisinin, and paclitaxel [2–4]. Even with their numerous applications, however, the usage of these enzymes as biocatalysts in industrial processes is still limited. Often, the low activity and multi-component electron-transport systems of these enzymes mean that their use is challenging and results in poor productivity outcomes.

The type I mitochondrial/bacterial CYP system is predominant in prokaryotes [5]. In this system, the electrons that are required for CYP reactions are delivered from NAD(P)H via ferredoxin (FDX) and ferredoxin reductase (FDR) (Fig. 1A) [6]. Although various orthologs of FDX and FDR are present in other bacterial strains, the heterologous reconstruction of the electron-transport partners in other host systems is often ineffective [7,8], suggesting that the employment of the most appropriate electron-transport partners is critical to obtain high CYP activity; of special concern is the variety of electron-transport mechanisms that bacterial CYPs are known to use. Electrons always move from NAD(P)H to flavoproteins in these pathways, and various bacterial CYPs accept electrons from either flavoproteins or FDX proteins (which receive them from FDR) [9]. The efficiency of CYP bioconversion largely depends upon its interaction with its redox partners and the interaction within the redox partners themselves; although, while several FDX and FDR are available in a genome of an organism, only a few compatible partners can function at full efficiency. One of the very well-known examples is the interaction of putidaredoxin (PDX) with putidaredoxin reductase (PDR) and CYP101A1 in *Pseudomonas putida*, which was shown to be quite specific [10]; thus, several experimental methods have been applied to determine compatible CYP redox partners. Performing the yeast two-hybrid system and chemical fixation using 1-ethyl-3-[(3-dimethylamino) propyl]-carbodiimide has been previously used to find the compatible redox partner [11,12], but all of these experimental procedures are time-consuming and expensive to undertake.

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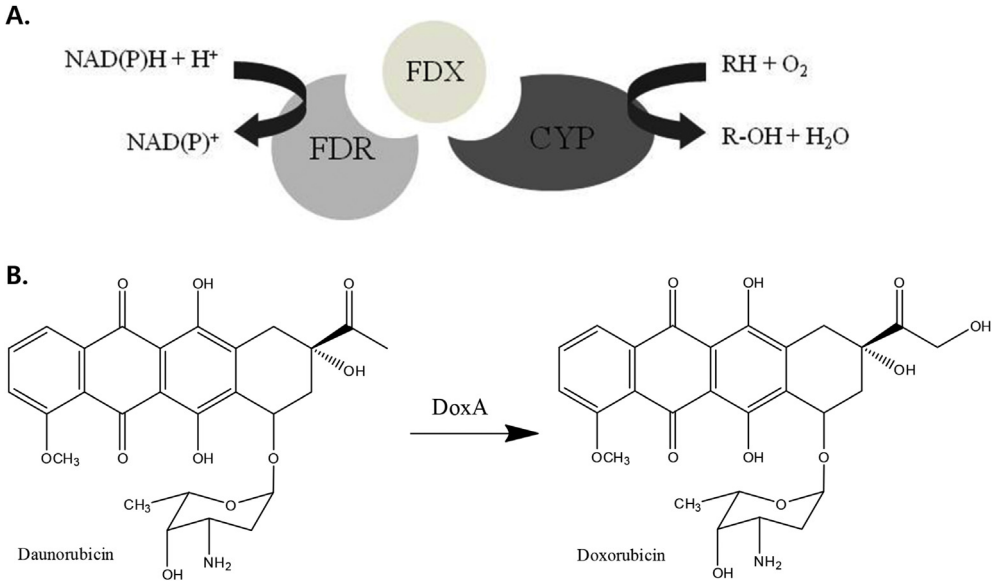

Fig. 1. The mechanism of bacterial type I CYP-related electron-transport system (A) and biosynthetic pathway catalyzed by DoxA (B).

In CYP research, a long-sought-after practical goal is the capitalization of the specificity of these enzymes in regio- and stereo--selection reactions for the production of chemicals that are difficult to prepare by traditional organic synthesis; however, the prerequisite for rational enzyme engineering is a knowledge of their structure. Crystallization of all CYPs is often laborious and costly both in terms of time and money, so a homology model can therefore be created to attain deeper insights into their structure and function; later, a rational mutant can be designed to directly influence the specific reaction as desired. In recent years, homology modeling has become a promising tool for the study of CYP functionality [13].

Streptomyces peucetius is known to produce the anthracycline drug doxorubicin (DXR), a potent anticancer drug. The final three-step hydroxylation for the production of DXR is carried out using CYP129A2 (DoxA) (Fig. 1B). The *S. peucetius* genome has 6 FDXs and 7 FDRs, but the real DoxA redox partner has not yet been identified [14]. To find the real redox partner of DoxA, we applied the *in silico* protein–protein docking software Z-Dock, and the R-dock software that was developed by Chen [15] and distributed by Accelrys. We over-expressed in *Escherichia coli* and purified DoxA along with three potential electron-transport proteins including two FDXs (FDX1 and FDX3) and one FDR (FDR2) from *S. peucetius*. We performed the enzymatic assay of DoxA using daunorubicin (DNR) as the substrate. We compared the production level between the native redox partners and the redox partner (PDX and PDR) from *P. putida*; on the basis of our result, we can establish the primary electron-transport pathway of DoxA.

2. Materials and methods

2.1. Protein structure

All of the protein structures of DoxA, FDXs, and FDRs used in the protein–protein docking were built using the "build homology model" within Accelrys Discovery Studio (DS) 3.5. The templates extracted from the Protein Data Bank (PDB, www.pdb.org) were aligned with the target and examined for conserved sequences. The protein model was generated using MODELER, which was originally developed by Sali [16].

2.2. Protein–protein docking by Z-Dock

The docking of the DoxA-FDXs and FDXs-FDRs were completed using Z-Dock, which is a fast, initial-stage algorithm for unbound and rigid-body docking. Z-Dock is a fast fourier transform (FFT)-based method that uses the Pairwise-Shape Complementarity (PSC) function that computes the total number of receptor-ligand atom pairs within a distance cut-off, minus a clash penalty for the identification of docked conformations. The best docked poses are evaluated using score hits based on atomic contact energies, desolvation, and electrostatic parameters [15]. The Z-Dock was performed for the Z-DocK poses within 60 clusters with a distance cut-off of 6 Å from the partner protein; after Z-Dock, we chose clusters with low Z-Rank-score poses.

2.3. Protein–protein refining by R-Dock

The best docked poses obtained from Z-Dock were further subjected to R-Dock, which is an effective algorithm for refining unbound predictions generated by the rigid-body docking algorithm that is Z-DocK. The main component of R-DocK is a three-stage energy minimization scheme, followed by evaluations of the electrostatic and desolvation energies. The ionic side chains were kept neutral in the first stages of minimization and were riveted to their full-charge stages in the last stage of the brief minimization [17]. R-Dock reranks (refines) the Z-Dock hits based on the multi-staged CHARM energy minimization method. The advantage of R-Dock is that it removes clashes and optimizes polar and charge interactions.

2.4. Calculation of interaction energy on the DoxA-FDXs and FDXs-FDRs

Interaction energy, which is the sum of the van der Waals and electrostatic energy of proteins, was calculated after the fixed-strain-backbone minimization of the docked complex was performed. The minimization protocol consisted of 20,000 steps of a smart minimize-minimization algorithm with an RMS gradient of 0.01.