BBA - Proteins and Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

Modulation of the interaction between human P450 3A4 and *B. megaterium* reductase *via* engineered loops☆

Silvia Castrignanò[1], Serena D'Avino[1], Giovanna Di Nardo, Gianluca Catucci, Sheila J. Sadeghi, Gianfranco Gilardi*

Department of Life Sciences and Systems Biology, University of Torino, Via Accademia Albertina 13, Torino, Italy

ARTICLE INFO

Keywords:
Cytochrome P450 3A4
Chimeric enzymes
Molecular lego
Drug-drug interaction

ABSTRACT

Chimerogenesis involving cytochromes P450 is a successful approach to generate catalytically self-sufficient enzymes. However, the connection between the different functional modules should allow a certain degree of flexibility in order to obtain functional and catalytically efficient proteins.

We previously applied the molecular Lego approach to develop a chimeric P450 3A4 enzyme linked to the reductase domain of P450 BM3 (BMR). Three constructs were designed with the connecting loop containing no glycine, 3 glycine or 5 glycine residues and showed a different catalytic activity and coupling efficiency. Here we investigate how the linker affects the ability of P450 3A4 to bind substrates and inhibitors. We measure the electron transfer rates and the catalytic properties of the enzyme also in the presence of ketoconazole as inhibitor.

The data show that the construct 3A4-5GLY-BMR with the longest loop better retains the binding ability and cooperativity for testosterone, compared to P450 3A4. In both 3A4-3GLY-BMR and 3A4-5GLY-BMR, the substrate induces an increase in the first electron transfer rate and a shorter lag phase related to a domain rearrangements, when compared to the construct without Gly. These data are consistent with docking results and secondary structure predictions showing a propensity to form helical structures in the loop of the 3A4-BMR and 3A4-3GLY-BMR.

All three chimeras retain the ability to bind the inhibitor ketoconazole and show an IC_{50} comparable with those reported for the wild type protein. This article is part of a Special Issue entitled: Cytochrome P450 biodiversity and biotechnology, edited by Erika Plettner, Gianfranco Gilardi, Luet Wong, Vlada Urlacher, Jared Goldstone.

1. Introduction

Among bio-analytical approaches suitable for biotechnological investigation, the availability of molecular systems mimicking the complexity of biomolecules structure-function implications is one of the most important requirements. This is particularly relevant when dealing with biochemical systems represented by multi-domain redox enzymes for which there is a high level of organization and differentiation of functions related to electron transfer, interaction with redox partners and catalytic activity. The so-called molecular Lego approach [1] allows the modular assembly of multi-domain enzymes in which the domain of proteins having the desired functional or structural properties are chosen as building blocks for the genetic construction of chimeric enzymes. In this paper, we provide a characterization of a cytochrome P450 3A4 based chimeric enzyme, with properties optimized in terms of electron transfer and catalysis.

The involvement of P450 3A4 in the metabolism of a wide range of drugs, hormones, chemicals and xenobiotics is well established and largely documented. The pharmacological effect of P450 3A4 metabolism can involve pro-drug activation [2], drug metabolism and side effects of the metabolites [3], drug-drug interaction [4]. The most clinically relevant effect on drug administration that can be ascribed to P450 3A4 metabolism is drug-drug interaction since it can induce the potentiation of the effect of one drug despite the other and lead to an increase of toxic side effect and/or the ineffectiveness of the therapy. One of the most important issues relevant to drug-drug interaction of

☆ This article is part of a Special Issue entitled: Cytochrome P450 biodiversity and biotechnology, edited by Erika Plettner, Gianfranco Gilardi, Luet Wong, Vlada Urlacher, Jared Goldstone.

* Corresponding author at: Department of Life Sciences and Systems Biology, University of Turin, Via Accademia Albertina 13, Torino 10123, Italy.
E-mail address: gianfranco.gilardi@unito.it (G. Gilardi).

[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.bbapap.2017.07.009
Received 12 June 2017; Received in revised form 9 July 2017; Accepted 17 July 2017

P450 3A4 activity is related to chemicals that are able to inhibit P450 3A4 reducing its ability to metabolise any co-administered pharmaceutical [5].

Due to the large amount of drugs that can actually interact with P450 3A4 enzyme, and to the continuous need to develop and to test new drugs, there is a high necessity for fast, accurate and versatile methods that could be reliably used for the screening of drug-drug interactions related to P450 3A4 activity.

Our recent research has been focused on the development of cytochrome P450 (P450) based fusion proteins obtained by the genetic linkage of P450 catalytic module with bacterial reductase module, in order to develop multi-domain chimeric P450 enzymes with high efficient electron transfer, catalytic and coupling properties [6–9]. The use of bacterial reductase domain instead of mammalian NADPH-dependent cytochrome P450 reductase (CPR), on which human P450 enzymes rely as electron supplier, has been found to be more useful since P450 fusion proteins with mammalian reductases require detergents, phospholipids and cytochrome b_5 to function [10]. Although several P450 chimeric enzymes have been successfully developed by fusing human P450 catalytic domain to bacterial reductase modules [10,11], a further improvement of this protein engineering strategy should not underestimate the importance of domains mutual flexibility that underlies coupling and catalysis efficiency in multi-domain enzymes. In this regard, it has been postulated that, similarly to rat CPR [12], in *Bacillus megaterium* flavocytochrome P450 reductase domain (BMR) FAD and FMN modules are connected by a hinge loop that is responsible for their arrangement in a closed conformation, allowing FAD to FMN electron transfer, and in an open conformation, allowing FMN to P450 haem electron transfer [13].

In the recent past, our group has set up different protein engineering approaches in order to develop a P450 chimeric enzyme with improved electron transfer capability, coupling and catalytic efficiency [10]. In particular, P450 3A4 was linked to different bacterial reductase modules showing improved turnover rate and coupling efficiency, but only when linked to BMR reductase domain P450 3A4 was able to use NADPH reducing equivalents in solution [8,11]. Very recently, we described a 3A4-BMR construct that was engineered in three variants by modulating the length of the peptide loop connecting the P450 3A4 catalytic domain and the BMR reductase domain [14]. The major findings related to the characterisation of these 3A4-BMR fusion proteins highlighted the importance of the inter-domain flexibility in order to obtain a high efficient electron transfer system. In particular, a better performance in terms of catalytic activity and coupling efficiency was detected for the chimeric system engineered with the longest loop.

Here we report the detailed characterisation of these three chimeric P450 3A4 enzymes: 3A4-BMR, 3A4-3GLY-BMR and 3A4-5GLY-BMR, engineered with a loop containing no glycine, 3 glycine and 5 glycine residues, on molecular docking of the two domains of the chimeric proteins, binding properties in the presence of testosterone and ketoconazole, and testosterone 6β-hydroxylation catalytic activity in the presence of NADPH regenerating system. Finally, we investigated 3A4-BMR fusion proteins inhibition of testosterone metabolism by ketoconazole in order to evaluate the chimeric system feasibility for the screening and characterisation of drug-drug interaction processes related to P450 3A4 activity.

2. Material and methods

2.1. Chemicals

All chemicals were of analytical grade and were purchased from Sigma Aldrich. All solutions were prepared just before use in ultra pure deionized water.

2.2. Molecular docking studies of 3A4-BMR fusion proteins

The crystal structure of P450 3A4 (2.05 Å resolution, PDB 1TQN) [15] and that of bacterial CYP BM3 FMN domain (2.03 Å resolution, PDB 1BVY) [16] were docked using the High Ambiguity Driven protein-protein DOCKing software (HADDOCK) [17–19]. The output of docking computational analysis was examined and the best structure in terms of Z-score was investigated in order to calculate the distance between FMN and haem redox centers using UCSF Chimera software. UCSF Chimera was also used for electrostatic potential investigation and representation. The three different loops connecting the two domains were manually built and energy minimized using Amber force field [20,21].

A prediction of the secondary structure composition of the loops connecting haem and FMN domains of 3A4-BMR chimeras molecular models was obtained using PSIPRED Protein Sequence Analysis Workbench server [22].

2.3. 3A4-BMR fusion proteins expression and purification

3A4-BMR fusion proteins, engineered following the strategy previously described [14] were heterologously expressed in competent *E. coli* DH5α cells using the corresponding plasmid vectors and purified by double step ion exchange chromatography as described by Dodhia et al. [10]. The enzyme purity was confirmed by loading purified protein samples on sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli's method [23] on 7.5% gel using a Mini-Protean apparatus (Bio-Rad, USA). Protein bands were observed after Coomassie blue staining.

2.4. Stopped flow experiments

Reduction of 3A4-BMR catalytic domain from ferric to ferrous-CO complex was investigated using a Hi-Tech scientific SF-61 single mixing stopped-flow system (TgK Scientific, UK) at 25 °C in a glovebox (Belle Technology, UK) with an oxygen concentration below 10 ppm. The reduction of 3A4-BMR haem domain was monitored at 450 nm following ferrous haem complexation with CO for 2250 s under anaerobic conditions both in absence and in presence of substrate, erythromycin or testosterone, in saturating concentration. Proteins and reducing agent solutions were saturated with CO and transferred to the sample handling unit syringes of the stopped-flow instrument. The experiment was performed by mixing the NADPH (syringe A, 200 μM NADPH in 50 mM potassium phosphate buffer pH 8.0) and the enzyme solutions (syringe B, 1 μM enzyme in 50 mM potassium phosphate buffer pH 8.0). Substrate was added in syringe B in saturating amount. Analysis of the resulting kinetic data was performed with Kinetic studio V3 software (TgK Scientific, UK). In order to calculate 3A4-BMR haem reduction rate, the plot of absorbance at 450 nm versus time was fitted to a single or double exponential function.

2.5. 3A4-BMR ligand binding spectroscopic titration

3A4-BMR binding to both testosterone substrate and ketoconazole inhibitor was investigated spectrophotometrically. Spectral changes observed upon ligand binding to 3A4-BMR chimeric enzymes were monitored by 8453E UV-VIS spectrophotometer (Agilent Technologies, CA) equipped with 1 cm path length cuvette. Ligand titration was carried out in 50 mM potassium phosphate pH 8.0 buffer at 37 °C using 500 μL of 1 μM protein solution. In the case of testosterone, ligand concentration was increased up to 300 μM and the binding was observed by following the enhancement in absorbance at 395 nm and the decrease in absorbance of the Soret peak at 418 nm (Type I shift) [24]. Binding saturation curves were constructed by plotting A_{395}–A_{418} spectral difference versus testosterone concentration. The resulting curves were fitted to Hill's equation for cooperative binding and the