Accepted Manuscript

Thermodynamics of interactions between mammalian cytochromes P450 and b5

Evgeny Yablokov, Anna Florinskaya, Alexei Medvedev, Gennady Sergeev, Natallia Strushkevich, Alexander Luschik, Tatsiana Shkel, Irina Haidukevich, Andrei Gilep, Sergey Usanov, Alexis Ivanov

PII: S0003-9861(16)30484-2

DOI: 10.1016/j.abb.2017.02.006

Reference: YABBI 7439

To appear in: *Archives of Biochemistry and Biophysics*

Received Date: 5 December 2016

Revised Date: 20 February 2017

Accepted Date: 21 February 2017

Please cite this article as: E. Yablokov, A. Florinskaya, A. Medvedev, G. Sergeev, N. Strushkevich, A. Luschik, T. Shkel, I. Haidukevich, A. Gilep, S. Usanov, A. Ivanov, Thermodynamics of interactions between mammalian cytochromes P450 and b5, *Archives of Biochemistry and Biophysics* (2017), doi: 10.1016/j.abb.2017.02.006.

Title page

Thermodynamics of interactions between mammalian cytochromes P450 and b5

Evgeny Yablokov [a,*], Anna Florinskaya [a], Alexei Medvedev [a], Gennady Sergeev [b], Natallia Strushkevich [b], Alexander Luschik [b], Tatsiana Shkel [b], Irina Haidukevich [b], Andrei Gilep [b], Sergey Usanov [b], Alexis Ivanov [a]

[a] Institute of Biomedical Chemistry, Pogodinskaya str. 10, bldg 8, Moscow, 119121, Russia

[b] Institute of Bioorganic Chemistry, Kuprevicha str. 5/2, Minsk, 220141, Belarus

* E-mail address: evgeyablokov1988@mail.ru

List of abbreviations: CYP - cytochrome P450; CYB5 - cytochrome b5; CYB5A and CYB5B – microsomal and mitochondrial isoforms of b5; SPR - surface plasmon resonance; RU - resonance units; Kd - equilibrium dissociation constant; ΔG, ΔH, ΔS - changes of the Gibbs free energy, enthalpy and entropy; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDC - 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl; NHS - N-hydroxysuccinimide; EPR - endoplasmic reticulum; SEM - standard error of the mean

Abstract

Cytochromes P450 (CYPs) play an important role in the metabolism of xenobiotics and various endogenous substrates. Being a crucial component of the microsomal monooxygenase system, CYPs are involved in numerous protein-protein interactions. However, mechanisms underlying molecular interactions between components of the monooxygenase system still need better characterization. In this study thermodynamic parameters of paired interactions between mammalian CYPs and cytochromes b5 (CYB5) have been evaluated using a Surface Plasmon Resonance (SPR) based biosensor Biacore 3000. Analysis of 18 pairs of CYB5-CYP complexes formed by nine different isoforms of mammalian CYPs and two isoforms of human CYB5 has shown that thermodynamically these complexes can be subdivided into enthalpy-driven and entropy-driven groups. Formation of the enthalpy-driven complexes was observed in the case of microsomal CYPs allosterically regulated by CYB5 (CYB5A-CYP3A4, CYB5A-CYP3A5, CYB5A-CYP17A1). The entropy-driven complexes were formed when CYB5 had no effect on the CYP activity (CYB5A-CYP51A1, CYB5A-CYP1B1, CYB5B-CYP11A1). Results of this study suggest that such interactions determining protein clustering are indirectly linked to the monooxygenase functioning. Positive ΔH values typical for such interactions may be associated with displacement of the solvation shells of proteins upon clustering. CYB5-CYP complex formation accompanied by allosteric regulation of CYP activity by CYB5 is enthalpy-dependent.

Keywords:

Cytochrome P450, Cytochrome b5, SPR, Optical biosensor, Thermodynamics, Protein-protein interactions

1. Introduction

Cytochromes P450 (CYPs) form a superfamily of monooxygenases, widely represented in living organisms. The cytochrome P450-dependent monooxygenase systems are present in practically all living organisms. Protein components of these systems are structurally conserved as shown by experiments on chimeric complexes formation between proteins isolated from different species of living organisms [1-3]. This is due to the fact that in spite of some species differences in the structure of protein partners, regions which are responsible for the intermolecular interaction have a similar structure.

In humans, more than fifty CYPs have been identified [4]; they are involved in various biochemical processes such as metabolism of xenobiotics (CYP3A4, CYP3A5, CYP2C9 and CYP1B1 [5- 8]), including metabolism of more than 50% of all known drugs, and activation of many pro-carcinogens (CYP1B1 [9]) as well as biosynthesis of steroid hormones and cholesterol (CYP51A1 [10], CYP11A1 [11], CYP17A1 [12], CYP11B1 and CYP11B2 [13]).

A small hemoprotein, cytochrome b5 (CYB5), is often involved in the CYP monooxygenase systems as an effector [14-19]. In humans two major isoforms of membrane bound CYB5 have been found; they are localized in the endoplasmic reticulum (type CYB5A) and in the outer mitochondrial membrane (type CYB5B) [20]. In erythrocytes a soluble splice variant of CYB5A has been also found [21].

At present the molecular mechanisms of protein-protein interactions in P450 monooxygenase systems are not well understood, mainly due to difficulties in experimental investigation of such membrane proteins. Therefore, researchers often use computer-aided molecular modeling allowing to build simplified models of complex formation between protein partners of interest and to estimate the energy of its formation (mainly enthalpy component in relative units) [22, 23]. The reliability of such computer models depends on available experimental results used as input data for modeling and choice of computational

methods and algorithms. In addition, a serious disadvantage of this approach is the almost complete lack of evaluation of the entropy component [24]. Such models of molecular complexes represent only hypotheses that need experimental verification including obtaining data on the affinity and thermodynamics of intermolecular interactions.

Recent progress in understanding of principles of protein-protein interactions is associated with employment of new molecular techniques. In the context of the microsomal monooxygenase system, complex formation by its constituents has been successfully studied by means of optical biosensors using surface plasmon resonance (SPR) [25]. This resulted in real time recording of intermolecular interactions and characterization of their equilibrium, kinetic and thermodynamic parameters.

The aim of this study was comparative analysis of thermodynamic parameters of the interaction of 9 different isoforms of mammalian CYPs with two isoforms of human CYB5 (CYB5A and CYB5B). For 18 different CYB5-CYP complexes the values of equilibrium dissociation constants (Kd), changes of the Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (-TΔS) were calculated. Analysis of obtained data showed that all examined CYB5-CYP complexes can be classified into two groups: (1) the enthalpy-dependent complexes of CYB5 with microsomal CYPs, which are regulated allosterically by CYB5; (2) entropy-dependent complexes of CYB5 with CYPs, which are insensitive to allosteric regulation by CYB5. Obtained data expand fundamental knowledge of protein-protein interactions in P450 monooxygenase systems.

2. Materials and Methods

2.1 Proteins

Nine different isoforms of recombinant mammalian CYPs (bovine CYP11A1; horse CYP17A1; human CYP2C9, CYP1B1, CYP51A1, CYP3A4, CYP3A5, CYP11B1,

CYP11B2) and two isoforms of human CYB5 (CYB5B and CYB5A) of high purity (> 95% by SDS-PAGE) have been used in this study. Methods of their expression in *E. coli* cells as hexahistidine tagged proteins, isolation, puirification, and functional analysis have been described earlier [19, 26-31]. Table 1 shows the differences between CYP sequences used by us and canonical deposited in Uniprot database. Before SPR-based experiments all proteins stored at -70ºC were functionally active.

[Table 1 should be here]

2.2 Reagents

Special reagents for the biosensor measurements were obtained from GE Healthcare (USA). These included buffer HBS-EP+ (150 mM NaCl, 3 mM EDTA, 0.05% surfactant P-20, 10 mM HEPES, pH 7.4), HBS-N buffer (150 mM NaCl, 10 mM HEPES, pH 7.4), 50 mM NaOH solution, 70% glycerol solution, 10 mM acetate buffer (pH 4.5 and 5.0), reagent kit for the covalent immobilization of proteins on the surface of the optical chip: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (EDC), N-hydroxysuccinimide (NHS), 1M ethanolamine-HCl (pH 8.5). Other reagents of analytical grade were obtained from local suppliers.

2.3 Equipment

The study of protein-protein interactions was performed on a SPR biosensor Biacore 3000 (GE Healthcare, USA) in the temperature range from 10°C to 40°C using standard CM5 chips coated with the carboxylated dextran layer. Real-time registration of intermolecular interactions was carried out in the form sensorgrams representing biosensor signal record in time (in resonance units, RU, 1 RU = 1 pg of protein; see below).

2.4 Immobilization of CYB5 on surface of the optical chip & SPR analysis of CYB5-CYP interactions

Both isoforms CYB5 (CYB5A and CYB5B) were immobilized by forming a covalent

bond between the carboxyl groups of carboxymethyl dextran on the surface of the optical chip and accessible amino groups of the hemeprotein. The immobilization procedure was performed using HBS-N as working buffer 1. The chip surface was initially washed with working buffer for 10 min at a flow rate 5 μl/min followed by short injection of 10 mM NaOH (for 1 min at at a flow rate 50 μl/min). Carboxyl groups of the dextran chip were activated by injecting a mixture of 0.2 M EDC and 0.05 M NHS for 7 min at a flow rate 50 μl/min. Immobilization of CYB5 was performed by injecting freshly prepared solution of this protein (20-40 μg) in immobilization buffer (10 mM sodium acetate, pH 4.5) for 10 min at a flow rate 2 μl/min. Unreacted carboxyl groups were then deactivated by injecting 1 M ethanolamine (pH 8.5) for 0.5 min at a flow rate 10 μl/min. The amount of the immobilized CYB5 estimated by the biosensor signal was in the range of 3-8 ng (300 - 500 fmol).

The interaction of nine different isoforms of CYPs with immobilized CYB5A or CYB5B was investigated in the concentration range from 50 nM to 5 μM and in the temperature range from 10°C to 40°C using HBS-EP+ as working buffer 2. Solutions containing increased CYP concentrations were sequentially injected into all biosensor channels. A SPR signal from the first channel (without immobilized CYB5) was subtracted from SPR signals obtained from channels with immobilized proteins. After injection of analytes (solutions of proteins of interest) for 7-10 min at a flow rate 5 μl/min, the chip surface was washed with working buffer 2 for 5-10 min at a flow rate 5 μl/min for registration of dissociation of protein complexes formed. After sensorgram registration the chip surface was recovered by injecting regeneration buffer (1 M NaCl, 0.2% CHAPS, 10 mM HEPES, pH 7.4) for 1 min at a flow rate of 50 μl/min. After injection of regeneration buffer the chip surface was washed with working buffer 2 for 15 min at a flow rate 5 μl/min. Nonspecific adsorption was evaluated by injecting 15 μM solutions of albumin and hemoglobin for 15 min at a flow rate 5 μl/min. These experiments demonstrated lack of interaction of albumin and hemoglobin with

immobilized CYB5B. The lack of interaction between immobilized CYB5 with injected hemoglobin suggests that the immobilized CYB5 exists mainly as a holo-protein as the apo-CYB5 can bind heme of hemoglobin [26]. As an example, Fig. 1 shows a typical series of sensorgrams of CYP3A4 interaction with immobilized CYB5A at 25°C. Calculation of kinetic parameters (rate constants of complex association and dissociation) and the equilibrium dissociation constant (Kd) was performed using BIAevaluation v. 4.1 software (GE Healthcare, USA).

[Fig. 1 should be here]

The Kd values were calculated for each temperature tested. Using temperature dependence of Kd values obtained for protein-protein complexes (Fig. 2) it is possible to generate Vant-Hoff plots (equations 1 and 2) as dependences of lnKd versus 1000/T,

$$Kd = \left(\frac{\Delta \mathrm{H}}{\mathrm{R}}\right) * \left(\frac{1000}{T}\right) - \left(\frac{\Delta S}{R}\right) \quad (1)$$

$$\Delta G = RT \ln Kd \quad (2),$$

where Kd is equilibrium dissociation constant, T is absolute temperature, ΔH is enthalpy change, ΔS is entropy change, R – the Boltzmann gas constant.

Enthalpy changes (ΔH) were calculated by multiplying the R constant for the angle coefficient of the straight line equation approximating the Vant-Hoff plot (correlation coefficient $r^2 \geq 0.93$).

[Fig. 2 should be here]

Changes on Gibbs free energy (ΔG) were calculated using equation (2), where Kd is equilibrium dissociation constant at 298°K. The -TΔS value was calculated using equation (3):

$$\Delta G = \Delta H - T\Delta S \quad (3).$$

Typically, each experiment for each analyzed pair of proteins was repeated three times using newly prepared chips with freshly immobilized ligands.

3. Results

Table 2 summarizes results of SPR measurements of interactions between nine different isoforms of CYPs with two isoforms of CYB5. Comparative analysis of the obtained thermodynamic parameters of the protein-protein interactions revealed existence of two groups of CYB5-CYP complexes: enthalpy-driven and entropy-driven complexes (Fig. 3).

[Table 2 should be here]

The enthalpy-driven complexes were formed during interaction of microsomal CYP3A4, CYP3A5 and CYP17A1 with both CYB5 isoforms (CYB5A or CYB5B). These complexes were characterized by negative values of the enthalpic term and positive values of the entropic term of ΔG ($\Delta H < 0$, $-T\Delta S > 0$). Obviously, a significant change in ΔH may be attributed to the electrostatic interactions, formation of salt bridges and hydrogen bonds [32]. It is known that CYB5 is an allosteric effector exactly for these CYPs and for this action electrostatic interactions are critical [33, 34]. In this case the entropic term of the Gibbs free energy change (ΔG) is positive ($-T\Delta S > 0$). This means that the complex formation probably involves polar groups for desolvation during displacement of water molecules from the contact area of proteins [35].

[Fig. 3 should be here]

The formation of entropy-driven complexes was observed in the case of CYB5 interaction with other examined CYPs (mitochondrial CYP11A1, CYP11B1, CYP11B2 and microsomal CYP1B1 and CYP51A1). Formation of these complexes was characterized by negative values of the entropic term ($-T\Delta S < 0$); this may be attributed to hydrophobic interactions and desolvation of the hydrophobic regions in the contact area [32, 35]. In this context, positive values of the enthalpic term ($\Delta H > 0$) can be determined by conformational changes in both CYP and CYB5 molecules [32].

Table 2 also shows that microsomal CYP1B1 and CYP51A1 form entropy-driven

complexes with both isoforms of CYB5 (CYB5A and CYB5B). At the same time, it is known that CYB5 has no effect on the activity CYP1B1 and CYP51A1 [36, 37], and the fact of their interaction without affecting the function of CYPs remains unclear. It can be assumed that such non-functional complexes are formed due to the interaction of hydrophobic parts of these CYPs with the hydrophobic membrane domain of CYB5. As opposed to previous observations, we have not detected the formation of complexes between microsomal CYP2C9 and CYB5 (Table 2). At the same time, several studies have shown a stimulating effect of CYB5A on the activity of CYP2C9 [36, 38]. It is possible that CYB5 transfers electrons to CYP2C9 via a shuttle mechanism with the formation of short-lived protein-protein complexes with dissociation rate constant > 1 s^{-1} [39].

4. Discussion

SPR analysis of 18 CYB5-CYP pairs performed in this work, allowed us to obtain the values of the equilibrium dissociation constants simultaneously with the values characterizing changes in enthalpy and entropy.

Comparative thermodynamic analysis of binary interactions between the components of mammalian monooxygenase systems (nine isoforms of CYP and two isoforms of CYB5) showed that the resulting CYB5-CYP complexes could be divided into two groups: the enthalpy-driven and entropy-driven complexes. Enthalpy-driven complexes are characteristic of the microsomal CYPs (CYP3A4, CYP3A5 and CYP17A1), which are allosterically regulated by CYB5. This allosteric regulation results in enhanced activity of CYP3A4, CYP3A5 towards selective substrates (e.g. testosterone, estrone, nifedipine, midazolam, amitriptyline, ellipticine) [15, 40-43] and it switches CYP17A1 17α-hydroxylase activity to 17/20-lyase [12, 44, 45]. Thus, the enthalpy-driven interactions of CYPs with CYB5 play a crucial role in the regulation of xenobiotic metabolism and androgen synthesis [44, 45].

The entropy-driven complexes of CYB5 with different CYPs (both microsomal and mitochondrial) are formed when CYB5 has no effect on the catalytic activity of CYP. In vitro interaction of proteins, located in different intracellular compartments (microsomal CYP and mitochondrial CYB5), may be attributed to conserved amino acid sequences typical for the monooxygenase CYP-dependent systems. For example, mitochondrial and microsomal CYB5 share structural similarity in their hydrophilic domains; they are also characterized by conserved residues required for interaction with CYPs [31]. The phenomenon of the entropy-driven complex formation can be explained on the structural basis of the monooxygenase systems, which generally include several protein components (cytochrome P450, cytochrome b5, NADPH-cytochrome b5 reductase, NADPH-cytochrome P450 reductase, adrenodoxin reductase and adrenodoxin or any other ferredoxin). Most of these proteins have hydrophobic loci needed for their interaction with the membrane [46]. Diffusion of membrane proteins is limited by the membrane plane and functioning of multienzyme complexes requires controlled lateral diffusion. Hydrophobic loci of protein components of the monooxygenase systems are able to interact not only with the membrane, but also with each other thus causing formation of protein clusters [46].

Since our experiments have been carried out in solution, direct interaction between CYPs and CYB5 reported here, raised several important questions: (i) how will the interactions be different when they have place in the EPR (endoplasmic reticulum) membrane; (ii) will the Kd values be affected? (iii) whether behavior of interacting proteins in 3D-space in solution will be as in (largely) 2-dimensional interactions in the membrane? (iv) will other membrane components influence the interactions recognized in this study? Although direct answer to these issues requires employment of additional experimental approaches, certain evidence exists in the literature that knockout of hepatic CYB5A from mice expressing CYP3A4 resulted in a decrease in the clearance of several substrates [47].

This suggests importance of the enthalpy driven allosteric interaction between CYB5A and CYP3A4, recognized in this study.

5. Conclusion

Results of this study suggest that protein-protein interactions determining protein clustering are indirectly linked to the monooxygenase functioning. Positive ΔH values typical for such interactions may be associated with displacement of the solvation shells of proteins upon clustering. CYB5-CYP complex formation accompanied by allosteric regulation of CYP activity by CYB5 is enthalpy-dependent.

Acknowledgements

This study was performed within the framework of the Program of Basic Scientific Research of Russian National Science Academies for 2013-2020.

References

[1] P.S. Stayton, M.T. Fisher, S.G. Sligar, Determination of cytochrome b5 association reactions. Characterization of metmyoglobin and cytochrome P-450cam binding to genetically engineered cytochrome b5, J. Biol. Chem. 263 (1988) 13544-13548.

[2] P.S. Stayton, T.L. Poulos, S.G. Sligar, Putidaredoxin competitively inhibits cytochrome b5-cytochrome P-450cam association: a proposed molecular model for a cytochrome P-450cam electron-transfer complex, Biochemistry 28 (1989) 8201-8205.

[3] P.S. Stayton, S.G. Sligar, Cytochrome P-450cam binding surface defined by site-directed mutagenesis and electrostatic modeling, Biochemistry 29 (1990) 7381-7386.

[4] F. P. Guengerich, Z. L. Wu, and C. J. Bartleson, Function of human cytochrome P450s: Characterization of the orphans, Biochem. Biophys. Res. Commun. 338 (2005) 465–469.

[5] A. L. Shen, K. A. O'Leary, and C. B. Kasper, Association of Multiple Developmental

Defects and Embryonic Lethality with Loss of Microsomal NADPH-Cytochrome P450 Oxidoreductase, J. Biol. Chem. 277 (2002) 6536–6541.

[6] D. W. Nebert, and D.W. Russell, Clinical importance of the cytochromes P450, Lancet 360 (2002) 1155–1162.

[7] I. Akiyama, K. Tomiyama, M. Sakaguchi, M. Takaishi, M. Mori, M. Hosok, S. Nagamori, N. Shimizu, N. Huh, M. Miyazaki, Expression of CYP3A4 by an immortalized human hepatocyte line in a three-dimensional culture using a radial-flow bioreactor. Int. J. Mol. Med. 14 (2004) 663-668.

[8] A.E. Rettie, J.P. Jones, Clinical and toxicological relevance of CYP2C9: drug-drug interactions and pharmacogenetics, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 477–94.

[9] S. Badal, R. Delgoda, CYP1B1: friend or foe? A critical review. OA Biochemistry 1(1) (2013) 8.

[10] G.I. Lepesheva, and M.R. Waterman, Sterol 14alpha-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms, Biochim. Biophys. Acta. 1770(3) (2007) 467-77.

[11] I. Hanukoglu, Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis, J. Steroid. Biochem. Mol. Biol. 43(8) (1992) 779–804.

[12] O.L. Guryev, A.A. Gilep, S.A. Usanov, R.W. Estabrook, Interaction of apo-cytochrome b5 with cytochromes P4503A4 and P45017A: relevance of heme transfer reactions, Biochemistry. 40(16) (2001) 5018-31.

[13] T. Kawamoto, Y. Mitsuuchi, K. Toda, Y. Yokoyama, K. Miyahara, S. Miura, T. Ohnishi, Y. Ichikawa, K. Nakao, H. Imura, Role of steroid 11 beta-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans, Proc. Natl. Acad. Sci. U.S.A. 89(4) (1992) 1458–62.

[14] O.V. Gnedenko, E.O. Yablokov, S.A. Usanov, D.V. Mukha, G.V. Sergeev, T.V. Bulko,

A.V. Kuzikov, N.E. Moskaleva, V.V. Shumyantseva, A.S. Ivanov, A.I. Archakov, SPR and electrochemical analyses of interactions between CYP3A4 or 3A5 and cytochrome b5, Chemical Physics Letters 593 (2014) 40–44.

[15] H. Yamazaki, T. Shimada, M.V. Martin, and F.P. Guengerich, Stimulation of cytochrome P450 reactions by apo-cytochrome b5: evidence against transfer of heme from cytochrome P450 3A4 to apo-cytochrome b5 or heme oxygenase. J. Biol. Chem. 276 (2001) 30885-91.

[16] S . Yamaori, H . Yamazaki, A. Suzuki, A . Yamada, H. Tani, T. Kamidate, Ki Fujita, T. Kamataki, Effects of cytochrome b(5) on drug oxidation activities of human cytochrome P450 (CYP) 3As: similarity of CYP3A5 with CYP3A4 but not CYP3A7, Biochem. Pharmacol. 66(12) (2003) 2333-40.

[17] T. Aoyama, K. Nagata, Y. Yamazoe, R. Kato, E .Matsunaga, H.V. Gelboin, F.J. Gonzalez, Cytochrome b5 potentiation of cytochrome P-450 catalytic activity demonstrated by a vaccinia virus-mediated in situ reconstitution system, Proc. Natl. Acad. Sci. U S A 87(14) (1990) 5425-9.

[18] M.P. Duarte, B.B. Palma, A.A. Gilep, A . Laires, J.S. Oliveira, S.A. Usanov, J. Rueff, M. Kranendonk, The stimulatory role of human cytochrome b5 in the bioactivation activities of human CYP1A2, 2A6 and 2E1: a new cell expression system to study cytochrome P450 mediated biotransformation, Mutagenesis 20(2) (2005) 93-100.

[19] A.A. Gilep, R.W. Estabrook and S.A. Usanov, Molecular cloning and heterologous expression in E. coli of cytochrome P45017alpha. Comparison of structural and functional properties of substrate-specific cytochromes P450 from different species, Biochemistry (Mosc.) 68 (2003) 86-98.

[20] T. Ogishima, J.Y. Kinoshita, F. Mitani, M. Suematsu, A. Ito, Identification of outer mitochondrial membrane cytochrome b5 as modulator for androgen synthesis in Leydig cells, J. Biol. Chem. 278(23) (2003) 21204-11.

[21] K. Abe, S. Kimura, R. Kizawa, F.K. Anan, Y. Sugita, Amino acid sequences of cytochrome b5 from human, porcine, and bovine erythrocytes and comparison with liver microsomal cytochrome b5, J Biochem 97(6) (1985) 1659-1668.

[22] V.S. Skvortsov, N.V. Belkina, A.S. Ivanov, Cytochrome P450 52A3: modelling of 3D structure and surface mutations, Molecular Simulation 24 (2000) 369-378.

[23] N.V. Belkina, M. Lisurek, A.S. Ivano, Bernhardt R. Modelling of three-dimensional structures of cytochromes P450 11B1 and 11B2, J. Inorg. Biochem. 87(4) (2001), 197-207.

[24] X. Cheng, I. Ivanov, Molecular dynamics. Methods Mol Biol. 929 (2012) 243-85.

[25] Y. Higashimoto et al, Involvement of NADPH in the interaction between heme oxygenase-1 and cytochrome P450 reductase, J. Biol. Chem. 280(1) (2005) 729–737

[26] A.A. Gilep, O.L. Guryev, S.A. Usanov and R.W. Estabrook, Apo-cytochrome b5 as an indicator of changes in heme accessability: preliminary studies with cytochrome P450 3A4, J. Inorg. Biochem. 87 (2001) 237-44.

[27] N. Strushkevich, S.A. Usanov and H.W. Park, Structural basis of human CYP51 inhibition by antifungal azoles. J. Mol. Biol. 397 (2010) 1067-78.

[28] S.A. Usanov, S.E. Graham, G.I. Lepesheva, T.N. Azeva, N.V. Strushkevich, A.A. Gilep, R.W. Estabrook and J.A. Peterson, Probing the interaction of bovine cytochrome P450scc (CYP11A1) with adrenodoxin: evaluating site-directed mutations by molecular modeling, Biochemistry 41 (2002) 8310-20.

[29] N. Strushkevich, F. MacKenzie, T. Cherkesova, I. Grabovec, S. Usanov and H.W. Park, Structural basis for pregnenolone biosynthesis by the mitochondrial monooxygenase system, Proc. Natl. Acad. Sci. USA 108 (2011) 10139-43.

[30] I.V. Haidukevich, A.A. Gilep, T.S. Cherkesova, S.A. Usanov, Cloning, heterologous expression, isolation and purification of the cytochrome human CYP2C9*1, CYP2C9*2 and CYP2C9*3, Proc. Natl. Acad. Sci. Bel., Ser. Chem. Sci. 3 (2012) 105–111.

[31] G.V. Sergeev, A.A. Gilep, and S.A. Usanov, The role of cytochrome b5 structural domains in interaction with cytochromes P450, Biochemistry (Moscow) 79 (2014) 406-416.

[32] E.S. Wesley, Protein−Protein Interactions: Interface Structure, Binding hermodynamics, and Mutational Analysis, Chem. Rev. 97 (1997) 1233-1250.

[33] C. Zhao, Q. Gao, A.G. Roberts, S.A. Shaffer, C.E. Doneanu, S. Xue, D.R. Goodlett, S.D. Nelson, and W.M. Atkins, Cross-Linking Mass Spectrometry and Mutagenesis Confirm the Functional Importance of Surface Interactions between CYP3A4 and Holo/Apo Cytochrome b5, Biochemistry 51 (2012) 9488–9500.

[34] H.M. Peng and R.J. Auchus, The Action of Cytochrome b5 on CYP2E1 and CYP2C19 Activities Requires Anionic Residues D58 and D65, Biochemistry 52 (2013) 210–220.

[35] G.P. Brady, K.A. Sharp, Entropy in protein folding and in protein—protein interactions, Curr. Opin. Struct. Biol. 7(2) (1997) 215-21.

[36] H. Yamazaki, M. Nakamura, T. Komatsu, K. Ohyama, N. Hatanaka, S. Asahi, N. Shimada, F.P. Guengerich, T. Shimada, M. Nakajima and T. Yokoi, Roles of NADPH-P450 Reductase and Apo- and Holo-Cytochrome b5 on Xenobiotic Oxidations Catalyzed by 12 Recombinant Human Cytochrome P450s Expressed in Membranes of Escherichia coli, Protein. Expr. Purif. 24 (2002) 329-37.

[37] D.C. Lamb, N.N. Kaderbhai, K. Venkateswarlu, D.E. Kelly, S.L. Kelly and M.A. Kaderbhai, Human Sterol 14a-Demethylase Activity Is Enhanced by the Membrane-Bound State of Cytochrome b5, Archives of Biochemistry and Biophysics 395(1) (2001) 78-84.

[38] T.D. Porter, The roles of cytochrome b5 in cytochrome P450 reactions, J. Biochem. Mol. Toxicol. 16(6) (2002) 311-316.

[39] Q. Bashir, A.N. Volkov, G.M. Ullmann & M.Ubbink, Visualization of the Encounter Ensemble of the Transient Electron Transfer Complex of Cytochrome c and Cytochrome c Peroxidase, J. Am. Chem. Soc. 132 (2010) 241–247.

[40] M.W. Voice, Y. Zhang, C.R. Wolf, B. Burchell and T. Friedberg, Effects of human cytochrome b5 on CYP3A4 activity and stability in vivo, Arch. Biochem. Biophys. 366 (1999) 116-24.

[41] A.A. Gilep et al., An enzymatically active chimeric protein containing the hydrophilic form of NADPH-cytochrome P450 reductase fused to the membrane-binding domain of cytochrome b5, Biochem. Biophys. Res. Commun. 284(4) (2001) 937-941.

[42] Z. Huang, F.P. Guengerich and L.S. Kaminsky, 16Alpha-hydroxylation of estrone by human cytochrome P4503A4/5, Carcinogenesis 19 (1998) 867-72.

[43] M. Stiborova et al., Ellipticine oxidation and DNA adduct formation in human hepatocytes is catalyzed by human cytochromes P450 and enhanced by cytochrome b5, Toxicology 302(2-3) (2012) 233-241.

[44] P. Lee-Robichaud, M.E. Akhtar, M. Akhtar, Control of androgen biosynthesis in the human through the interaction of Arg347 and Arg358 of CYP17 with cytochrome b5, Biochem. J. 332 (1998) 293–6.

[45] D.F. Estrada, J.S. Laurence, E.E. Scott, Substrate-modulated cytochrome P450 17A1 and cytochrome b5 interactions revealed by NMR, J. Biol. Chem. 288 (2013) 17008–17018.

[46] D.R. Davydov, Molecular Organization of the Microsomal Oxidative System: a New Connotation for an Old Term, Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry 10 (2016) 10-21.

[47] E.E. Scott, C.R. Wolf, M. Otyepka, S.C. Humphreys, J.R. Reed, C.J. Henderson, L.A. McLaughlin, M. Paloncýová, V. Navrátilová, K. Berka, P. Anzenbacher, U.P. Dahal, C. Barnaba, J.A. Brozik, J.P. Jones, D.F. Estrada, J.S. Laurence, J.W. Park, and W.L. Backes, The Role of Protein-Protein and Protein-Membrane Interactions on P450 Function, Drug. Metab. Dispos. 44 (2016) 576–590.

Figure/Table Legends

Fig. 1. An example of sensorgrams set of CYP3A4 interaction with immobilized CYB5A at 25°C. 1 – 3.3 μM, 2 – 2.0 μM, 3 – 1.0 μM, 4 – 0.75 μM, 5 – 0.5 μM, 6 – 0.35 μM, 7 – 0.2 μM.

Fig. 2. A – Sensorgrams of the enthalpy-driven interaction between immobilized CYB5B and CYP3A4, concentration of CYP3A4 was 0.5 μM; 1, 2, 3 – interactions at 10ºC, 25ºC, and 40ºC respectively. B - Sensorgrams of the entropy-driven interaction between immobilized CYB5B and CYP51A1, concentration of CYP51A1 was 0.5 μM; 1, 2, 3 – interactions at 10ºC, 25ºC, and 40ºC respectively. C – Vant-Hoff plots for CYB5B-CYP3A4 (a) and CYB5B- CYP51A1 (b).

Fig. 3. Allocation of CYB5-CYP complexes into groups, depending on the values of enthalpic (ΔH) and entropic (-TΔS) terms. Group of enthalpy-driven complexes of CYB5 with microsomal CYPs (CYP17A1, CYP3A4 and CYP3A5) is marked by oval.

Table 1. The entire of modification all sequence modifications is shown in the table below. Canonical sequences (full-length mature type) are shown in Uniprot database. All CYP's contain 6 his-tag, added at C-tail.

Table 2. The values of equilibrium dissociation constants (Kd, at 25°C), changing in Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (-TΔS) during complex formation of nine isoforms of mammalians CYPs with two isoforms of human CYB5.

*"no" – Complex formation was not observed.

Figures/Tables

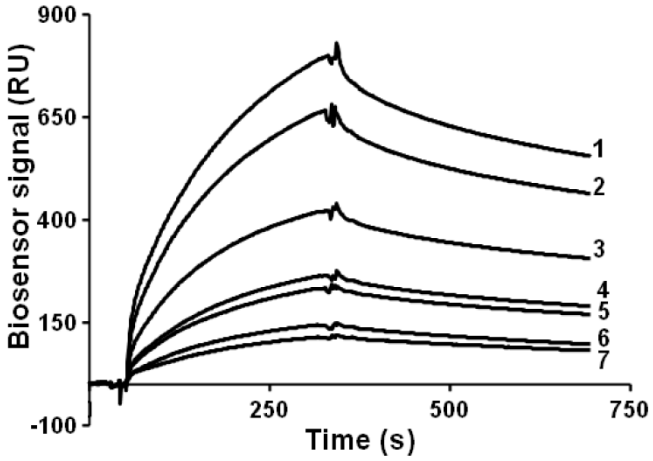

Fig. 1. [single-column]

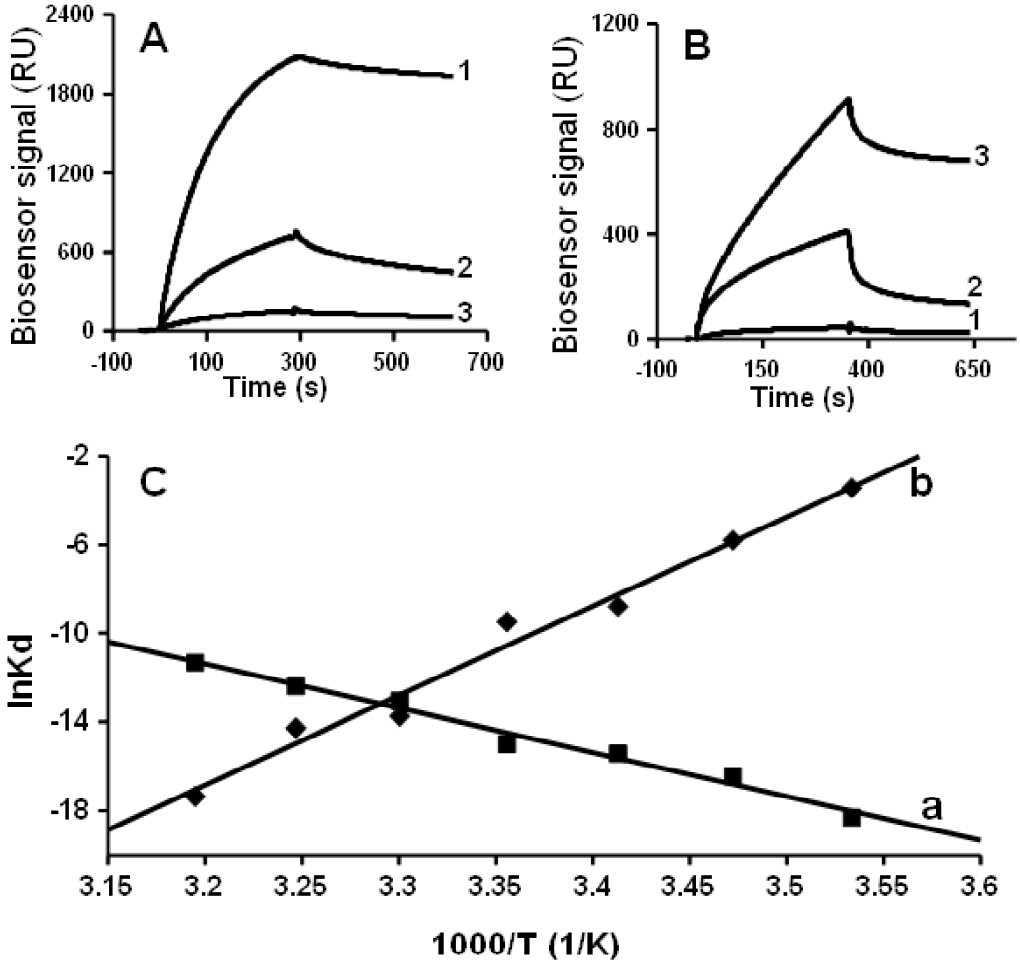

Fig. 2. [double-column]

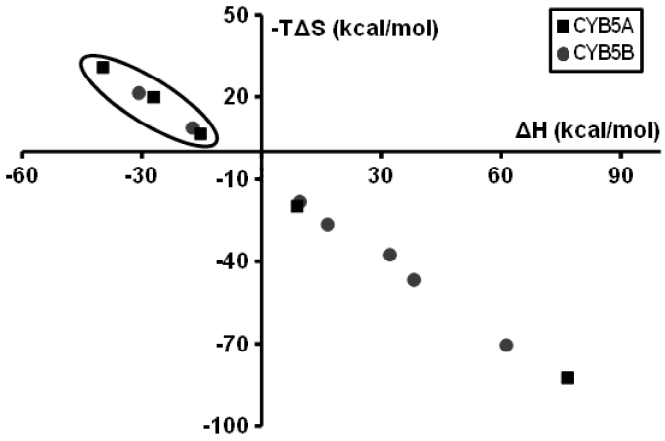

Fig. 3. [single-column]

Table 1. The entire of modification all sequence modifications is shown in the table below. Canonical sequences (full-length mature type) are shown in Uniprot database. All CYP's contain 6 his-tag, added at C-tail.

Isoform of cytochrome	Sequence modifications
CYP11A1 bovine	Full-length mature type
CYP17A1 horse	The part of N-terminal transmembrane anchor domain (residues 2–17) was deleted. The resulting second codon was replaced by GCT (Ala).
CYP2C9 human	The N-terminal transmembrane anchor domain (residues 1–23) was replaced with MAKKT-sequence.
CYP1B1 human	The part of N-terminal transmembrane anchor domain (residues 1–27) was replaced with MAKKTSS sequence.
CYP51A1 human	The N-terminal transmembrane anchor domain (residues 1–53) was replaced with MAKKT sequence.
CYP3A4 human	The part of N-terminal transmembrane anchor domain (residues 1–18) was replaced with MALLLAVF sequence.
CYP3A5 human	The part of N-terminal transmembrane anchor domain (residues 1–18) was replaced with MALLLAVF sequence.
CYP11B1 human	Full-length mature type
CYP11B2 human	Full-length mature type
CYB5B human	Full-length mature type
CYB5A human	Full-length mature type

Table 2. The values of equilibrium dissociation constants (Kd, at 25°C), changes in Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (-TΔS) during complex formation between nine isoforms of mammalians CYPs with two isoforms of human CYB5.

Complex CYB5-CYP	**Kd (μM)**	**ΔG (kcal/mol)**	**ΔH (kcal/mol)**	**-TΔS (kcal/mol)**
CYB5A-CYP3A4	0.35±0.04	-8.8±0.6	-39.7±4.0	30.7±4.6
CYB5B-CYP3A4	0.30±0.03	-8.9±0.8	-30.7±3.8	21.3±3.2
CYB5A-CYP3A5	4.8±0.4	-7.2±0.2	-27.0±1.8	19.8±2.9
CYB5B-CYP3A5	0.50±0.04	-8.6±0.2	-17.3±1.8	8.7±1.3
CYB5A-CYP51A1	75.3±6.8	-5.6±0.8	76.6±9.5	-82.3±12.3
CYB5B-CYP51A1	103.0±18.7	-5.4±0.8	32.1±3.8	-37.6±5.6
CYB5A-CYP2C9	no*	no	no	no
CYB5B-CYP2C9	no	no	no	no
CYB5A-CYP17A1	0.4±0.03	-8.8±0.7	-15.3±2.5	6.5±0.8
CYB5B-CYP17A1	no	no	no	no
CYB5A-CYP11A1	no	no	no	no
CYB5B-CYP11A1	0.50±0.02	-8.5±0.5	38.2±4.3	-46.7±5.8
CYB5A-CYP11B1	no	no	no	no
CYB5B-CYP11B1	0.5±0.03	-8.6±0.3	9.6±1.4	-18.1±3.2
CYB5A-CYP11B2	no	no	no	no
CYB5B-CYP11B2	0.19±0.003	-9.2±0.4	61.3±6.4	-70.5±6.7
CYB5A-CYP1B1	0.01±0.001	-10.8±1.2	8.9±0.9	-19.7±2.2
CYB5B-CYP1B1	0.04±0.005	-10.0±1.1	16.6±1.9	-26.6±2.3

Data represent mean ± SEM of three independent experiments.

*"no" – Complex formation was not observed.

Recombinant Cytochromes P450 (CYP) form complexes with cytochrome b5 (CYB5).

There are enthropy- and enthalpy-driven paired CYP-CYB5 complexes.

In enthalpy-driven complexes CYB5 acts as an allosteric regulator.