Mapping of interaction between cytochrome P450 2B4 and cytochrome b_5: the first evidence of two mutual orientations

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Abstract

OBJECTIVES: The cytochrome P450 (P450) and cytochrome b_5 are membrane hemoproteins composing together with flavoprotein NADPH:P450 reductase a mixed function oxidase (MFO) system. The knowledge of the interaction between P450 and its redox partners within a MFO system is fundamental to understand P450 reaction mechanism, an electron transport from its redox partner and also detoxification of xenobiotics and/or metabolism of endogenous substrates with all positive or negative aspects for organisms.
METHODS: The chemical cross-linking by soluble carbodiimide (EDC) in combination with the liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) has been employed to characterize the contact surface regions involved in the transient interaction between two catalytic domains of P450 2B4 and cytochrome b_5.
RESULTS: The cross-linking reaction was accomplished in an equimolar catalytic complex of P450 2B4:cytochrome b_5 and the covalent hetero-dimers detected on SDS-PAGE electrophoresis were analyzed (after in gel trypsin digestion) using LC-HRMS to identify cross-linked amino-acid residues. The computed in silico models of P450 2B4:cytochrome b_5 complex using amino-acids participating in cross-links (Asp134, Lys139, Glu424 and Glu439 located on a proximal surface of P450 2B4) suggest interpretation that two different types of cytochrome b_5 orientations are present in the studied interaction within a MFO system: the first allowing potential cytochrome b_5 electron donation to P450, the second one inducing cytochrome b_5 modulation of P450 structural changes.
CONCLUSIONS: The results demonstrated the capability of the used experimental approach to map the interaction between P450 and cytochrome b_5 suggesting the formation of multi-meric structures within a MFO system as interpretation of the two observed mutual orientations.

ORIGINAL ARTICLE

Abbreviations:

DLPC	- dilauroylphosphatidylcholine
EDC	- 1-ethyl-3-[3-dimethyl(aminopropyl)]carbodiimide hydrochloride
LC-HRMS	- liquid chromatography coupled with high resolution mass spectrometry
M	- mol/l
m/z	- mass/charge
MFO	- mixed function oxidase
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
P450	- cytochrome P450
ppm	- particle per million
SDS-PAGE	- sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCEP	- Tris(2-carboxyethyl) phosphine hydrochloride

INTRODUCTION

The cytochrome b_5 and cytochrome P-450 (P450) are membrane hemoproteins that compose together with flavoprotein NADPH:P450 reductase a mixed function oxidase (MFO) system localized in the endoplasmic reticulum (Coon 1978). The crucial function of this MFO system consists in the monooxygenation of mainly hydrophobic compounds resulting in detoxification of xenobiotics and/or metabolism of endogenous substrates. Unfortunately the detoxification of some xenobiotics is sometimes reversed to produce compounds more toxic/carcinogenic that are able to modify biomolecules such as DNA, RNA and proteins with all negative consequences for organisms (Guengerich 2005). Thus, P450s are known to be involved in the activation of some chemical carcinogens.

The knowledge of the interaction between P450 and its redox partners (NADPH:P450 reductase and cytochrome b_5) is fundamental to understand not only reaction mechanism of P450 and an electron transport from its redox partner but also to increase our knowledge about metabolism of xenobiotics with all positive or negative aspects for organisms. The cytochrome b_5 has been shown to stimulate, inhibit or have no effect on activity of P450s depending on the P450 isoform, the substrate, and experimental conditions (for references see review Zhang *et al.* 2005). In general, cytochrome b_5 has been suggested to act either as a mediator of electron flow to oxyferrous P450 (Bonfils *et al.* 1981) or as an allosteric modifier of the P450 system (Hlavica 1984) influencing the P450 reaction rate or even the pattern of P450 produced metabolites (Akhtar *et al.* 2005; Kotrbova *et al.* 2011). The studied rabbit P450 2B4 isoform is an orthologue to human P450 2B6 that is involved in oxidation of a number of structurally different carcinogens (e.g. nicotine, aflatoxin B1, styrene, and aminochrysene) and the drugs (e.g. diazepam, cyclophosphamide, iphosphamide, tamoxifen, antipyrine, and lidocaine) (for references see Stiborova *et al.* 2002).

The chemical cross-linking is becoming a valuable tool for the low resolution structure determination of protein complexes in their native states. In combination with mass spectrometry analysis of the cross-linked products it is possible to determine cross-linked residues in a binary complex of proteins in a reasonable time-scale using small quantities of proteins. Literature data prove this approach to be useful in solving the protein–protein interactions (for references see review Sinz 2006). One of the most used reagent is a "zero length cross-linker" 1-ethyl-3-[3-dimethyl(aminopropyl)] carbodiimide hydrochloride (EDC) that reacts with a carboxylic group on the first protein, forming an amine-reactive O-acylisourea intermediate that may condense with an amine on the second protein yielding a conjugate of the two molecules joined by a stable amide bond. This reaction results in covalent cross-link of these two proteins, without any part of EDC molecule remaining in the final conjugate (Thus, EDC is often called a "zero length cross-linker"). The amide bond formed by this reaction provides a neutral linkage of amino and carboxylic groups of two closely located or interacting amino-acid residues within distances of under 5 Å (Kalkhof *et al.* 2005).

Here we present an application of chemical cross-linking in reconstituted systems containing purified native proteins incorporated into the membrane. The combination of liquid chromatography and high resolution mass spectrometry (LC-HRMS) provided identification of amino-acid residues participating in the interface between cytochrome b_5 and P450 2B4.

MATERIAL AND METHODS

Chemicals

7-pentoxyresorufin (7-pentyl-7-hydroxy-3-H-phenoxazin-3-one), resorufin and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, USA). 1-ethyl-3-[3-dimethyl(aminopropyl)]carbodiimide hydrochloride (EDC), Coomassie Brilliant Blue R-250, dilauroylphosphatidylcholine (DLPC), iodoacetamide, Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and pyridine were from Fluka Chemical Co. (St. Louis, USA). Bicinchoninic acid was from Pierce (Rockford, USA), trypsin from Promega (Madison, USA), acetonitrile and water of LiChrosolv quality from Merck (Darmstadt, Germany). The other chemicals were obtained from Lachema (Brno, Czech Republic).

Purification of P450 2B4, cytochrome b_5 and NADPH:P450 reductase and their characterization

All proteins were purified from microsomes prepared from rabbit liver using previously described procedures (Sulc et al.; 2004, Sulc *et al.* 2010). The animal experiment was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which complies with the Declaration of Helsinki. Each protein was characterized by SDS-PAGE electrophoresis in combination with mass spectrometry analysis after trypsin digestion to confirm sequences of all pure proteins. The total P450 content was measured based on complex of reduced P450 with carbon monoxide (Omura & Sato

1964), the total NADPH:P450 reductase or cytochrome b_5 content were determined using absorbance of the purified protein at 455 nm or at 412 nm, respectively (Vermilion & Coon 1978). Protein concentrations were assessed using bicinchoninic acid and bovine serum albumin as a standard (Wiechelman *et al.* 1988).

O-dependentylation of 7-pentoxyresorufin by purified proteins in reconstituted system

The preparation of reconstituted system in DLPC vesicles as well as estimation of an optimal ratio of P450 2B4 and NADPH:P450 reductase were described previously (Sulc *et al.* 2010). Briefly, the optimal ratio of P450 2B4 and cytochrome b_5 was determined using P450 2B4 specific 7-pentoxyresorufin-*O*-dependentylase activity with 30 μM 7-pentoxyresorufin, 2 μM P450 2B4 and 2 μM NADPH:P450 reductase using a fluorescence spectrometer LS55 ($\lambda_{excitation}$ 530 nm, $\lambda_{emission}$ 588 nm, PerkinElmer, Waltham, USA) after 10 min incubation at 37 °C in 0.15 mM DLPC vesicles, 50 mM pyridine:HCl buffer (pH 6.5) and 150 mM sodium chloride. Three different P450 2B4:cytochrome b_5 ratios were tested (1:1, 1:2, and 1:4). Experiments with increasing concentration of sodium chloride (0, 50, 150, and 500 mM) were performed in the same system with P450 2B4, NADPH:P450 reductase and cytochrome b_5 in 1:1:1 molar ratio.

Cross-linking, separation, proteolysis of cross-linked products and LC-HRMS analysis

The cross-linking reaction with 0.05 mg/ml of EDC was carried out with 2 μM P450 2B4 and 2 μM cytochrome b_5 in reconstituted system containing 0.15 mM DLPC and 50 mM pyridine-HCl buffer (pH 6.5) for 10 hrs in final volume of 20 μl at 37 °C in a shaking incubator. The concentration of NaCl was adjusted in individual samples to 0, 50, 150 and 500 mM. The EDC cross-linking reaction was quenched by 2-mercaptoethanol present in electrophoresis sample buffer, and after boiling and centrifugation the sample was loaded on SDS-PAGE. The electrophoretically separated protein bands were visualized using 0.25% (w/v) Coomassie Brilliant Blue R-250. The excised protein spots from SDS-PAGE gel were de-stained, all cysteine residues in protein bands were modified using TCEP and iodoacetamide, and processed for MALDI-TOF mass spectrometry by in-gel digestion with trypsin as described previously (Sulc *et al.* 2010). The mixture of extracted peptides was applied on a peptide micro-trap microcolumn (Michrom Bioresources, Auburn, CA, USA) to remove any salt and buffer components prior to LC-HRMS analysis that was described together with MS data analysis previously (Haladova *et al.* 2012).

Protein-protein docking

In silico protein-protein docking of rabbit P450 2B4 with truncated N-terminal membrane part and soluble domain of rabbit cytochrome b_5 was performed using the flexible docking approach implemented in program HADDOCK with starting structures: a homologous model of truncated rabbit P450 2B4 (Hodek *et al.* 2004) and PDB coordinates 1DO9 from RCSB protein databank (Banci *et al.* 2000). This software utilizes information on predicted protein interfaces for ambiguous interaction restraints to drive the docking process. The recommended docking procedure is described in detail elsewhere (de Vries *et al.* 2010). Three approaches how to utilize the experimental data have been applied. In the first one, the docking calculation was performed using all P450 2B4 amino-acid residues (Asp134, Lys139, Glu424 and Glu439) determined in cross-links as active "anchors" of protein-protein interaction. Secondly, the identified covalent cross-link Glu424 (P450 2B4)-Lys24 (cytochrome b_5) was used as a distance constrain of protein-protein interaction. In the last approach, the two pairs of identified covalent cross-links, that do not mutually exclude, Glu424 (P450 2B4)-Lys24 (cytochrome b_5) and Glu439 (P450 2B4)-Lys19 (cytochrome b_5) were given as constrain distances of protein-protein interaction. For all three approaches all charged residues exposed on the both P450 2B4 and cytochrome b_5 surfaces were considered to be passively involved in this protein-protein interaction, no penalty was imposed if a passive residue was not directly involved in the mentioned interaction. In order to extend the number of individual mutual orientations of both proteins the default values of (i) number of structures for rigid body docking, (ii) number of trials for rigid body minimisation and (iii) number of structures for the explicit solvent refinement were doubled and the optional solvated docking was performed.

RESULTS AND DISCUSSION

The methods of chemical cross-linking and mass spectrometry were used for the mapping of protein interface between two membrane proteins, P450 2B4 and cytochrome b_5. To accomplish our goal, the highly purified and well characterized proteins (P450 2B4, cytochrome b_5 and for metabolic activity measurements NADPH:P450 reductase) were obtained from liver homogenate up to the electrophoretical homogeneity employing techniques preserving a native structure of these proteins. Every protein sample was characterized by MS analysis and also by determination of both protein and molar concentrations (for results see Table 1).

All purified proteins were fully active in the reconstituted system (in the P450 2B4 selective *O*-dependentylation of 7-pentoxyresorufin) with determined activity of 33.9±1.9 nmol resorufin/min.nmol P450 in the system with no cytochrome b_5 and phosphate buffer (pH 7.4). The activity determination in the above system at conditions used in chemical cross-linking experiments with optimal pH for EDC chemistry (pyridine buffer, pH 6.5 instead phosphate buffer, pH 7.4) revealed the value of 22.8±1.8 nmol resorufin/min.nmol P450 (100% P450

Tab. 1. Characterization of purified rabbit liver microsomal P450 2B4, cytochrome b_5 and NADPH:P450 reductase.

Quantity [Unit] Purified protein	Protein conc. [mg/ml]	Specific content [nmol/mg]	Identified sequence code	MASCOT score	Sequence coverage [%]	Matched/Searched peptides
P450 2B4	3.8±0.3	11.0	CP2B4_RABIT	288	46	26/28
cytochrome b_5	1.8±0.2	11.1	CYB5_RABIT	216	61	10/12
NADPH:P450 reductase	1.4±0.2	9.6	NCPR_RABIT	337	45	33/34

Numbers in the table are averages ± S.D. of three parallel experiments. Probability Based MASCOT Score is –10*Log(P), where P is the probability that the observed match is a random event. The scores greater than value 54 were significant (p<0.05). MALDI-TOF peak lists were searched against a SwissProt protein database subset of the Other mammalia taxonomy group using MASCOT™ software with the following settings: enzyme chemistry – trypsin, missed cleavages 1, carbamidomethyl modification of cysteine, variable single oxidation of methionine and peptide mass tolerance ± 50 ppm.

Tab. 2. Identified EDC cross-linked products between cytochrome b_5 and P450 2B4 by LC-HRMS.

$[M+H]^+$	Error [ppm]	P450 2B4 peptide	Cytochrome b_5 peptide
2479.2371	0.4	134–151 DFGMGKRSVEERIQEEAR	8–10 DVK
2504.2917	1.6	126–140 RFSLATMRDFGMGKR	19–24 KHNHSK
2657.4001	0.1	134–140 DFGMGKR	19–33 KHNHSKSTWLILHHK
1719.9058	1.0	435–443 IC(CAM)LGEGIAR	19–24 KHNHSK
3117.5918	1.2	423–434+ox–M NEGFM(ox)PFSLGKR	20–33 HNHSKSTWLILHHK

underlined amino-acids in peptide sequences label a reactive residue, M(ox) marks an oxidized methionine and C(CAM) means a cysteine modified with carbamidomethyl.

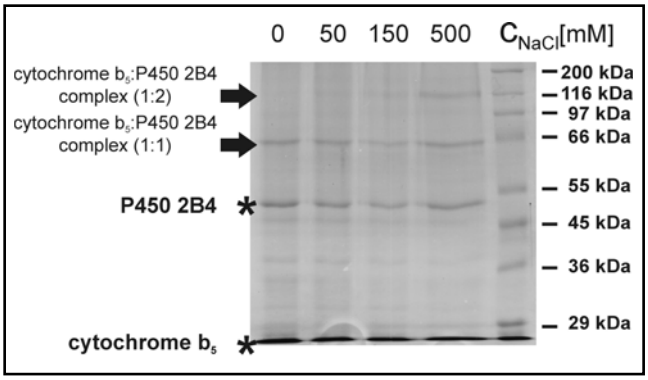

Fig. 1. Monitoring of cytochrome b_5:P450 2B4 heterodimer formation using EDC cross-linker in buffer with increasing concentration of sodium salt (0, 50, 150 and 500 mM) on 8% SDS-PAGE electrophoresis (Coomassie Brilliant Blue R-250 staining, Sigma wide-range molecular weight standards). Arrow labels the heterodimeric products and asterisk marks both monomers.

2B4 activity) demonstrating a decrease in P450 activity due to presence of pyridine buffer or lower pH value. Then the effect of added cytochrome b_5 was tested. Three molar ratios of P450 2B4:cytochrome b_5 (1:1, 1:2 or 1:4) disclosed 162%, 82%, or 44% of P450 2B4 activity, respectively. In agreement with literature (Zhang *et al.* 2007), the equimolar cytochrome b_5 concentration stimulated the P450 2B4 activity, but two or four fold molar excess of cytochrome b_5 inhibited *O*-dependentylase activity of P450 2B4, probably by competition of cytochrome b_5 at the NADPH:P450 reductase binding site on the P450 2B4 surface.

The chemical cross-linking reaction of cytochrome b_5:P450 2B4 complex using zero-length cross-linker EDC yielded the covalent heterodimeric products detectable on SDS-PAGE (labeled by arrow in the Figure 1). Similar experiment in presence of NADPH:P450 reductase in addition to cytochrome b_5 and P450 2B4 was also performed, but production of a cytochrome b_5:P450 2B4 covalent heterodimeric product was not affected (data not shown). The increasing salt content (0–500 mM of NaCl) did not prevent formation of the cytochrome b_5:P450 2B4 cross-linked product and the cytochrome b_5:P450 2B4 complex was still detectable even at the highest concentration (500 mM) of sodium chloride in consistence with metabolic activities. In addition to the control experiment with 150 mM NaCl (100%), three additional concentrations of NaCl were tested (0, 50 and 500 mM), yielding 98%, 118%, and 78% of 7-pentoxy resorufin O-depenthylase activity characteristic for P450 2B4, respectively. (All these experiments were carried out with P450 2B4 to cytochrome b5 molar ratio 1:1). In agreement with published data (Nadler & Strobel 1988; Schenkman *et al.* 1994), the results of both experimental approaches suggest not only the electrostatic character of the studied interaction but also a significant contribution of hydrophobic effects to the formation of the cytochrome b_5:P450 2B4 binary complex.

As we described previously, the incubation of a cross-linker with cytochrome b_5:P450 2B4 produced a mixture of covalently cross-linked heterodimers that probably included different amino-acid residues participating in novel covalent junction between both proteins. The proportion of these orientations statistically depends on presence of each species in the

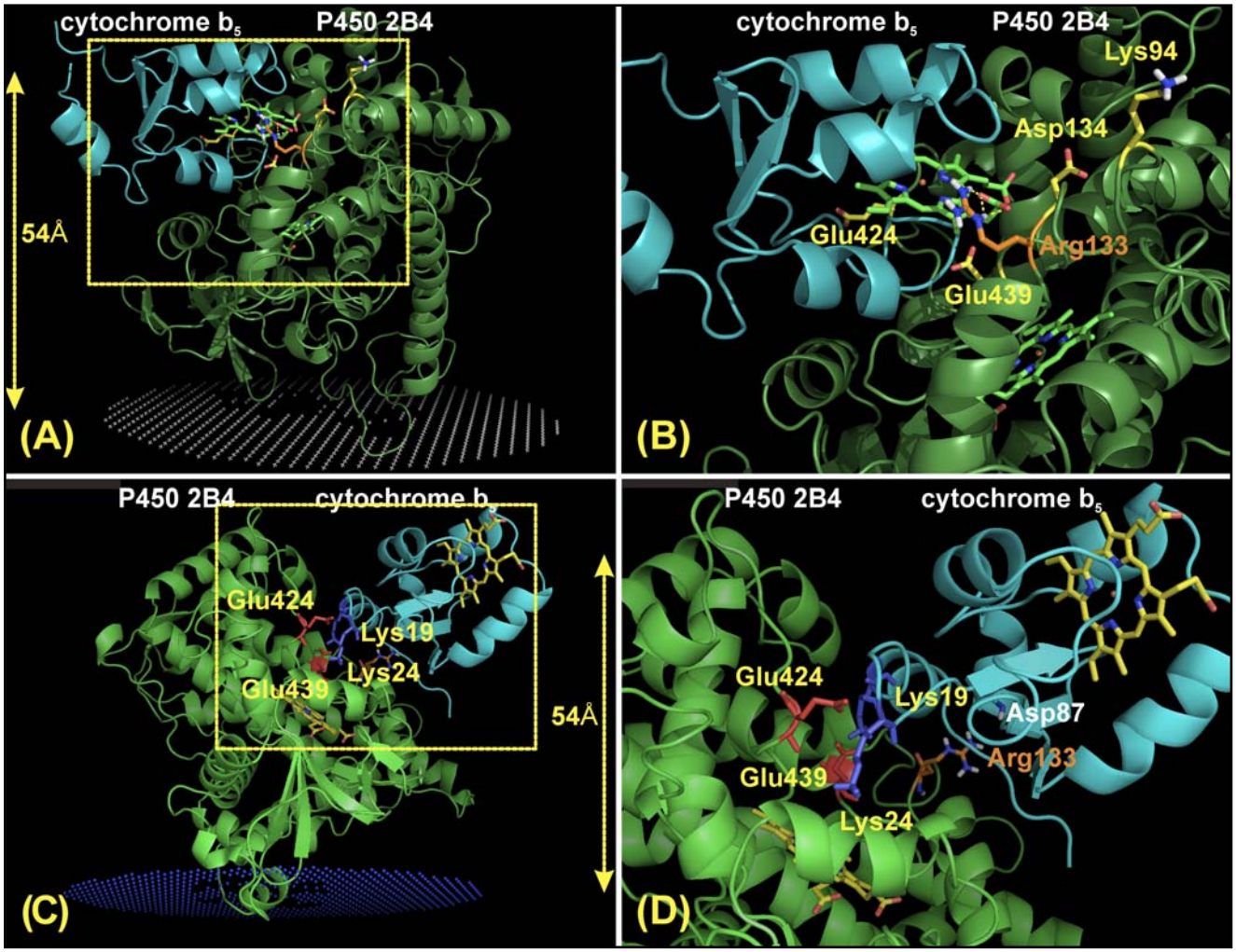

Fig. 2. The structure of the cytochrome b_5:P450 2B4 binary complex predicted by protein-protein docking calculations prepared using PyMOL: (A) using the EDC covalently cross-linked P450 2B4 amino-acid residues (Asp134, Lys139, Glu424 and Glu439) rendered as sticks (yellow), (B) its detailed view within the interface of this complex (the interaction of cytochrome b_5 heme propionate (green) with P450 2B4 Arg133 (orange) is represented as dashed line), (C) using two constrain distances Glu424 (P450 2B4)-Lys24 (cytochrome b_5) and Glu439 (P450 2B4)-Lys19 (cytochrome b_5), and (D) its detailed view within the interface of this complex (also the interaction of cytochrome b_5 heme Asp87 (cyan) with P450 2B4 Arg133 (orange) is shown). The membrane association of the P450 2B4 is positioned at the bottom.

reaction mixture. After quenching the cross-linking reaction, the SDS-PAGE electrophoresis was employed to separate both monomers (cytochrome b_5 and P450 2B4) from the protein band containing the mixture of varied heterodimeric orientations. The proteolysis of cross-linked species to peptides using trypsin, and the analysis of the resulting peptide mixture by mass spectrometry with a high accuracy allowed to determine the a unique combination of two cross-linked peptide sequences that fit to protease specificity (the trypsin generates peptides with C-terminal arginine or lysine) and also to the EDC reaction mechanism (coupling of amino and carboxylic groups). Table 2 presents (in each row) the experimentally observed *m/z* values, mass error of calculated and experimental *m/z* values (maximally 2 ppm) and sequences of the covalently cross-linked peptides. The calculated *m/z* value was determined as mass of any P450 2B4 trypsinised peptide plus mass of any cytochrome b_5 trypsinised peptide minus mass of water eliminated during the EDC cross-linking reaction. All identified peptide sequences permitted to deduce only one possible combination of the interacted and/or covalently coupled amino-acid residues (underlined amino-acid in Table 2).

Four P450 2B4 amino-acid residues: Asp134, Lys139, Glu424 and Glu439, have been found to participate in the interaction with cytochrome b_5. Interestingly, all four these residues are located on a P450 2B4 proximal surface. Because some of identified covalent cross-links are mutually incompatible and can not be present together in one orientation, we have performed *in silico* simulation of P450 2B4:cytochrome b_5 interaction taking into account four identified P450 2B4 residues (Asp134, Lys139, Glu424 and Glu439) and the whole

cytochrome b_5 surface. Semi flexible half-blind docking approach using contemporary modeling procedures implemented in program HADDOCK (Vries *et al.* 2010) was employed for such a study. The obtained structure shown in Figure 2A revealed the highest HADDOCK score –213.7±17.0 a.u. with a high contribution of electrostatic interactions to the resulting score. These findings are in a good agreement with predominant stabilization of the protein-protein complex by complementary charged residues suitably pre-organized on the contact interface (Im & Waskell 2011; Bridges *et al.* 1998). Our results of chemical cross-linking experiments supported by metabolic assay results studying the effect of increasing ion strength (0, 50, 150, and 500 mM) suggested also a significant contribution of hydrophobic effects to the cytochrome b_5:P450 2B4 binary complex formation. This argument is supported by the large calculated inter-molecular contact area (the buried surface area in the P450 2B4 and cytochrome b_5 binary complex was calculated as 2050±80 Å^2). Probably the extensive and tight van der Waals contacts in this interaction domain can protect the ionic pairs formed from the access of a solvent and in this way stabilize the protein complex against the disruption by high salt concentrations. Although we cannot perform the protein-protein docking in the system associated with a membrane directly, the relative orientation of the P450:cytochrome b_5 binary complex to the membrane can be deduced. We used the proposed membrane orientation available in OPM database (Lomize *et al.* 2006) and obtained approximately the 54 Å distance between a hydrophobic layer of the membrane and C-terminus of cytochrome b_5 in the calculated binary complex (Figure 2A). This distance is lower than potential length of 22 amino acids (a segment between the C-terminal residue of the used soluble domain of rabbit cytochrome b_5 (model 1DO9) and the first hydrophobic residue of its proposed membrane anchor (Trp109)) that allows in a parallel β-sheet structure the maximal distance of 75 Å (3.4 Å/residue). Surprisingly, this docking model suggests the distance of 3.42 Å between nitrogen atom of the lysine amino-group (cytochrome b_5 Lys24) and carbon atom of the glutamic acid carboxylic group (P450 2B4 Glu424). This is in agreement with the proposed maximal distances of 5 Å for that the cross-linker EDC is able to connect amino and carboxylic acid groups (Kalkhof *et al.* 2005). Also the cytochrome b_5 heme propionate interaction with P450 2B4 Arg133 exposed on the P450 proximal surface was found (shown in Figure 2B). The role of P450 2B4 Arg133 was previously discussed suggesting the electrostatic pairing with the P450 heme propionates and movement of this Arg133 towards the proximal surface of P450 2B4 after the substrate/inhibitor binding into P450 cavity (Sulc *et al.* 2008). Therefore, this obtained orientation of the cytochrome b_5:P450 2B4 complex may reflect the cytochrome b_5 allosteric modulation of P450 metabolic activity or cytochrome b_5 mediation of electron flow to oxyferrous P450 reported in literature (Bonfils *et al.* 1981; Hlavica 1984). When the identified covalent cross-link Glu424 (P450 2B4)-Lys24 (cytochrome b_5) was used as an individual constrain distance of protein-protein interaction, almost the identical model was obtained (values of top structure: HADDOCK score was –159.1±11.0 a.u., the buried surface area was 1400±60 Å^2).

The identified cross-link Glu424 (P450 2B4)-Lys24 (cytochrome b_5) allows only one combination of two simultaneously presented pairs of constrain distances with another cross-link Glu439 (P450 2B4)-Lys19 (cytochrome b_5). The simulation of this interaction resulted in possible orientations fitting both two unique cross-links (Figure 2C). The proposed membrane orientation and a distance between a hydrophobic layer of the membrane and C-terminus of cytochrome b_5 using OPM database are acceptable in this calculated binary complex. But the heme of cytochrome b_5 is oriented out of the P450 2B4 proximal surface, the interaction P450 2B4 Arg133 with cytochrome b_5 Asp87 is presented in a detailed view in Figure 2D, and this P450 2B4:cytochrome b_5 interaction may result only in allosteric modulation of P450 metabolic activity.

Taking together our data, the existence of two mutual orientations of cytochrome b_5 and P450 in their complexes is presented. First, which is depicted in the Figure 2A, represents a commonly accepted cytochrome b_5 binding, when its heme is close to the proximal surface of P450. This orientation allows both cytochrome b_5 roles, the electron donation to P450 as well as the induction of cytochrome P450 structural changes, resulting in the stimulation of its catalytic activity. The second orientation, which takes into account two cross-links, suggests so far unpredicted position of cytochrome b_5 towards P450. In this orientation (shown in Figure 2C) the heme of cytochrome b_5 is exposed to solvent and its distance to P450 does not make any electron transfer possible. The presence of both these two cross-linked orientations found in the P450:cytochrome b_5 heterodimer represents probably only a part of more complex arrangement of both proteins in the membrane environment. This assumption is supported by the existence of a protein band at SDS-PAGE electrophoretic gel with molecular weight close to 116 kDa, probably containing two molecules of P450 and one of cytochrome b_5 (see Figure 1). Although a low yield of this cross-link did not allow more detailed analysis, the mass spectrometry analysis identified both P450 and cytochrome b_5 proteins in this band and supported our speculation that P450 and cytochrome b_5 can form multi-meric structures within the MFO system.

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Potential Conflicts of Interest: *None disclosed.*

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