

Quantification of interactions between cytochrome P450 2B4 and cytochrome b₅ in a functional membrane complex

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Abstract

OBJECTIVES: The mammalian mixed function oxidase (MFO) system participates in hydroxylation of many hydrophobic endogenous compounds as well as xenobiotics such as drugs and carcinogens. This biotransformation system, located in a membrane of endoplasmic reticulum, consists of cytochrome P-450 (P450), NADPH:P450 oxidoreductase and a facultative component, cytochrome b₅. The knowledge of the interactions among the individual components of the MFO system is essential to understand the relationships between the structure and function of this system that finally dictate a qualitative and quantitative pattern of produced metabolites (e.g. detoxified xenobiotics and/or activated carcinogens). To elucidate the quantitative aspects of the interactions within the MFO system we acquired the photo-initiated cross-linking approach.

METHODS: The photo-initiated cross-linking employing cytochrome b₅ as a protein nanoprobe [an amino acid analogue of methionine (pMet) was incorporated into cytochrome b₅ sequence during recombinant expression] was used to quantify its interaction with P450 2B4 in a functional membrane complex. The cross-linking was initiated by UV-irradiation that formed from a pMet photolabile diazirine group highly reactive carbene biradical. This biradical is able to covalently bind amino acids in the close proximity and to form cross-link. The Met 96 of cytochrome b₅ is situated in a linker region between its catalytic and membrane domains, while Met 126 and 131 are located in its membrane domain. The combination of several methods (electrophoresis in polyacrylamide gel, isoelectric focusing, Edman N-terminal degradation and amino acid analysis) was employed to characterize the molar ratio of P450 2B4 to cytochrome b₅ in formed covalent cross-links to quantify their transient interactions.

RESULTS: The successfully produced cytochrome b₅ nanoprobe (with confirmed pMet incorporation by mass spectrometry) stimulates the catalytic activity of P450 2B4 when reconstituted with NADPH:P450 oxidoreductase *in vitro* in dilauroylphosphatidylcholine (DLPC) vesicles. The cross-linking was carried out in similar reconstituted system without NADPH:P450 oxidoreductase, and at least three products were separated on 1D SDS-PAGE. The molar ratio

of P450 to cytochrome b₅ in each complex was estimated using the above-mentioned combination of methods as 1:1, 1:2 and 2:1.

CONCLUSIONS: The results demonstrate the utility of cytochrome b₅ nanoprobe to study the interactions in MFO system. Using this nanoprobe, heterodimer with P450 2B4 and in addition also heterooligomers were identified, suggesting rather complex interactions of both proteins in this system that suppose the formation of such multimeric structures in the membrane of endoplasmic reticulum.

Abbreviations:

DLPC	- dilauroylphosphatidylcholine
DMEM-LM	- Dulbecco's Modified Eagle Medium with absence of methionine and leucine
EDC	- 1-ethyl-3-[3-dimethyl(aminopropyl)]carbodiimide hydrochloride
EDTA	- ethylenediaminetetraacetic acid
m.u.	- mass unit
MS	- mass spectrometry
m/z	- mass/charge
MFO	- mixed function oxidase
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
OD _{600nm}	- optical density at wavelength 600 nm
P450	- cytochrome P450
SDS-PAGE	- sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCEP	- Tris(2-carboxyethyl) phosphine hydrochloride

INTRODUCTION

The photo-initiated cross-linking of protein nanoprobe is a “zero length cross-linking” experimental technique suitable for the determination of the assembly of various protein complexes in their native states (Ptáčková *et al.* 2014). A reasonable time-scales and consumption of relatively small quantities of protein are two major advantages of this approach that is an alternative to chemical cross-linking method (Sinz 2006). To determine structural information of the studied protein complexes, both cross-linking methods are usually combined with mass spectrometry (MS). When both cross-linking approaches are compared, the photo-initiated method is more powerful one due to the absence of limitations in reaction specificity and of restrictions of reaction conditions that are inherent to chemical cross-linkers (Ptáčková *et al.* 2014). To prepare the protein nanoprobe suitable for photo-initiated cross-linking, the selected amino acid residues from the sequence of protein of interest (e.g. Met or Leu) should be at least partially exchanged to respective analogues containing a photo-labile group (Suchanek *et al.* 2005). In the next step, the recombinant photo-labile protein nanoprobe has to be produced in a suitable expression system. The reconstitution, incubation and photo-activation of the studied proteins thereafter produce a mixture of covalently cross-linked oligomers. The abundance of cross-linked species, their ratio in these particular complexes and diverse protein orientations statistically depends on the incidence of corresponding transient interactions

in the reaction mixture. The applied UV-irradiation decomposes the diazirine functional group in the photo amino acid analogue and generates the highly reactive carbene biradical. It is able to bind to any amino acid residues within distances below 5 Å similarly to a “zero length chemical cross-linker” (Kalkhof *et al.* 2005). In the case of protein-protein interaction mapping, the next necessary steps are the separation of monomers and oligomers (e.g. by one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS-PAGE)), and their further characterization (e.g. by MS analysis).

The two membrane hemoproteins were studied, cytochrome P-450 (P450) and cytochrome b₅, are together with a flavoprotein NADPH:P450 reductase components of a mixed function oxidase (MFO) system located in the membrane of endoplasmic reticulum (Coon 1978). The hydroxylation of numerous hydrophobic compounds, the crucial function of the MFO system, results in: (i) detoxification of xenobiotics, (ii) metabolism of hydrophobic endogenous substrates, and/or (iii) activation of a variety of chemical carcinogens leading to formation of more toxic/carcinogenic metabolites that are able to modify biomolecules such as DNA, RNA and proteins (Guengerich 2005; Stiborova *et al.* 2013). During the P450-mediated monooxygenation reaction, the oxygen molecule is activated in the P450 active center by a transfer of two electrons from NADPH and/or NADH molecules catalyzed by NADPH:P450 reductase and/or NADH:cytochrome b₅ reductase with cytochrome b₅, respectively. While the transfer of electrons to P450 through NADPH:P450 reductase is supposed to be essential for a P450 reaction cycle, the role of cytochrome b₅ in this process is still rather enigmatic. The cytochrome b₅ 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). During its action, the P450 reaction rate can be influenced (stimulated, inhibited and not affected) by cytochrome b₅ (Schenkman & Jansson 2003; Kotrbova *et al.* 2009) and, moreover, even the pattern of metabolites produced by P450 might be modified by this protein (Kotrbova *et al.* 2011). The P450 2B4 enzyme studied in this work is a rabbit orthologue of human P450 2B6 that is involved in oxidation of a number of structurally different carcinogens (e.g. nicotine, aflatoxin B1, styrene, and aminochrysene) and drugs (e.g. diazepam, cyclophosphamide, iphosphamide, tamoxifen, antipyrine, and lidocaine) (for references see Stiborova *et al.* 2002).

Here we present an application of photo-initiated cross-linking in reconstituted systems assembled from the purified proteins (P450 2B4 and cytochrome b₅) *in vitro* incorporated into the artificial membrane of dilauroylphosphatidylcholine (DLPC)-mediated liposomes. The cytochrome b₅, which was used as a protein photo-initiated cross-linking nanoprobe, contains three methionines in its amino acid sequence. They were par-

tially substituted by a methionine analogue containing a photo-labile diazirine functional group (Suchanek *et al.* 2005). These methionine residues are found at position 96 that is located in linker domain and at the positions 126 and 131, which are situated within C-terminal transmembrane domain (Koberova *et al.* 2012). Using the combination of several techniques, the quantification of both enzymes, ratio of cytochrome b_5 and P450 2B4, participating in formation of oligomeric structures was determined.

MATERIAL AND METHODS

Chemicals

Amino acids and reagents for Dulbecco's Modified Eagle Medium with absence of methionine and leucine (DMEM-LM), bovine serum albumin, dithiotreitol (DTT), isopropyl β -D-1-thiogalactopyranoside (IPTG), acrylamide, bis-acrylamide, sodium dodecyl sulphate, trifluoroacetic acid, 7-pentoxoresorufin (7-pentyl-7-hydroxy-3-H-phenoxazin-3-one), and resorufin purchased from Sigma Chemical Co. (St. Louis, USA). Coomassie Brilliant Blue R-250, dilauroylphosphatidylcholine (DLPC), EDTA sodium salt, iodoacetamide, Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), and potassium phosphates were from Fluka Chemical Co. (St. Louis, MO, USA). Bicinchoninic acid and photo-methionine (L-2-amino-5,5-azi-hexanoic acid, pMet) were from Pierce (Rockford, IL, USA), acetonitrile and water of LiChrosolv quality from Merck (Darmstadt, Germany), DEAE-Sephacrose CL4B from GE Healthcare (Pittsburgh, PA, USA), OligoTM R3 Bulk Media from Applied Biosystems by Life Technologies (Grand Island, NY, USA), trypsin and chymotrypsin from Promega (Madison, WI, USA). All other chemicals were of analytical grade or better.

Purification of P450 2B4 (CP2B4_RABIT), cytochrome b_5 (CYB5_RABIT) and NADPH:P450 reductase (NCPR_RABIT) and their characterization

All proteins were prepared and purified by procedures described previously (Sulc *et al.* 2010). Each protein was characterized by combination of 1D SDS-PAGE with mass spectrometry (MS) after trypsin (for rabbit P450 2B4 and NADPH:P450 reductase) or chymotrypsin (for cytochrome b_5 after recombinant expression) digestion to confirm sequences of all pure proteins (Sulc *et al.* 2009). The total P450 content was measured as the complex of reduced P450 with carbon monoxide (Omura & Sato 1964), the total contents of NADPH:P450 reductase or cytochrome b_5 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). The preparation of reconstituted system in DLPC vesicles as well as estimation of an optimal ratio

of P450 2B4, cytochrome b_5 and NADPH:P450 reductase were described previously (Sulc *et al.* 2012).

Recombinant expression and purification of cytochrome b_5 protein nanoprobe

The recombinant protein expression was carried out in *E. coli* BL21-GOLD (DE-3) [genotype *E. coli* B F-ompT hsdS(r_B - m_B -)dcm+Tet^r gal^l (DE3) endA Hte] transformed with the expression vector pET22b. After growth at 37°C in LB-medium till OD_{600nm} value approximately 0.6 and rigorous washing by sterile 10 mM phosphate buffer (pH 7.4, at 6°C), the cells' cultivation continued over 1 hr at 37°C in DMEM-LM supplemented with photo-methionine analog (pMet, Pierce) and L-leucine after induction of cytochrome b_5 expression by isopropyl β -D-1-thiogalactopyranoside (1 mM final concentration) was added prior to the (Koberova *et al.* 2012). The cells were harvested by centrifugation, and lysed using sonication, the content of prosthetic groups was boosted by heme donation and microsomal fraction was solubilised by detergent. The protein was prepared by the combination of ion-exchange chromatography using the gradient of KCl concentrations for elution of proteins (DEAE-Sephacrose, GE Healthcare). Finally, rechromatography of the cytochrome b_5 nanoprobe for detergent substitution was performed as described previously (Kotrbova *et al.* 2009).

Photo-initiated cross-linking, separation, proteolysis of cross-linked products and MS analysis

The cross-linking reaction was carried out with 2 μ M P450 2B4 and 8 μ M cytochrome b_5 in a reconstituted system containing 0.3 mM DLPC and 50 mM phosphate buffer (pH 7.4). After DLPC vesicles preparation (sonication of DLPC film in a tube with buffer till clear solution using probe, 60 W) the both proteins were added and pre-incubated for 1 hr at 6°C in final volume of 20 μ l. The photo cross-linking was carried out at ambient temperature by UV-irradiation for 195 sec in a quartz tube (Oriol photolyser equipped with the Hg-Arc lamp emitting at 254 nm). The cross-linking reaction was quenched by 2-mercaptoethanol present in the electrophoresis sample buffer, and after boiling and centrifugation the sample was loaded on SDS-PAGE. The electrophoretically separated protein bands were visualized using silver staining. The distance between a center of each analysed protein band and front was determined by micrometry meter and obtained values were used for MW estimation after gel calibration using the determined mobilities of MW standards. The protein spots excised from the gel were de-stained, all cysteine residues in protein 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.* 2012). To remove any salts and buffer components, the mixture of extracted peptides was applied on a peptide micro-trap microcol-

umn (Michrom Bioresources, Auburn, CA, USA) prior to MS analysis together with the process of MS analysis as was described previously (Haladova *et al.* 2012).

Cytochrome b₅ nanoprobe-P450 cross-links quantification: 1D/2D-electrophoresis, N-terminal Edman degradation, amino acid composition analysis

The products of cross-linking reaction were separated employing 1D SDS-PAGE using 12% polyacrylamide gel or 2D SDS-PAGE combining 3-10NL IPG strip (13 cm, GE Healthcare, USA) with 10% polyacrylamide gel. For 1D SDS-PAGE, the gel was loaded with 20 µl of the cross-linking reaction mixture. In the case of 2D SDS-PAGE, 80 µl of cross-linking reaction mixture was precipitated with 500 µl of cold acetonitrile at -20 °C, and after centrifugation the whole pellet was dissolved in loading buffer. The both electrophoretic gels were visualized using silver staining. The N-terminal Edman degradation was performed with protein bands electroblotted onto the PVDF membrane at PROCISE 491 instrument (Applied Biosystems, Grand Island, NY, USA) according to instruction manual. The amino acid composition analysis was performed using hydrolysis by 4.7 N HCl at 110 °C for 24 hrs in a sealed glass tube for both samples of protein bands, pieces of destained polyacrylamide gel or incised PVDF membrane of electroblotted proteins visualized with Ponceau-Red (1% in water (w/v)). The hydrolyzed mixture of amino acids from proteins excised from a PVDF membrane was dried on Jouan RC 10.10. SpeedVac and reconstituted by vortexing in 20 µl of 20 mM constant-boiling HCl. After the reconstitution step, 60 µl AccQ Fluor Borate buffer and 20 µl AccQ Fluor reagent (Waters Corporation, MA, USA) were added and the amino acids were incubated at 55 °C for 10 min. A mixture of the derived amino acids was analyzed using Waters AccQ Tag column on Beckman Coulter HPLC Gold chromatograph with Merck-Hitachi F-1080 fluorescence detector (excitation 250 nm, emission 395 nm). Data were collected and evaluated employing Data Apex CSW32 chromatography software.

RESULTS AND DISCUSSION

The photo-initiated cross-linking was used for the mapping and quantification of protein-protein oligomers formed between two membrane proteins, P450 2B4 and cytochrome b₅. The *in vitro* reconstitution of highly purified and well characterized proteins (P450 2B4, cytochrome b₅ and for metabolic activity measurements also NADPH:P450 reductase) was used to accomplish our goal.

The recombinant cytochrome b₅ nanoprobe (protein concentration of 3.4 mg/ml, specific content of 66 nmol/mg of protein) was prepared from *E. coli* and electrophoretically homogenous protein was carried out (see Figure 1A). This final preparation of cytochrome b₅ was additionally characterized by its UV-VIS spectrum (see

Figure 1B). The characteristic absorbance maximum at 412 nm was used to determine its molar concentration. The decrease in absorbance at 280 nm was used to monitor elimination of the detergent. To confirm the identities and purity of all protein preparations, they were characterized by MS analysis (data not shown). In the case of recombinantly expressed cytochrome b₅, amount of incorporated pMet in all sites in the protein sequence was also determined comparing intensity of both peptides, with or without incorporated pMet (it was approximately 50%, see Figure 1C). The electrophoretically homogenous NADPH:P450 reductase and P450 2B4 (protein concentration 4.5 mg/ml, specific content 1.8 nmol/mg) were prepared as described previously (Sulc *et al.* 2012). All purified proteins in the reconstituted system were fully enzymatically active (measured as the P450 2B4 selective O-depentylation of 7-pentoxoresorufin), having activity of 33.9±1.9 nmol resorufin/min.nmol P450 (the value used as the control, 100% P450 2B4 activity) in the system without presence of cytochrome b₅ in 50 mM phosphate buffer (pH 7.4). The effect of added cytochrome b₅ to the system on P450 2B4 enzyme activity was also analyzed; three molar ratios of P450 2B4:cytochrome b₅, 1:1, 1:2 or 1:4, disclosed 182%, 265%, or 204% of P450 2B4 activity, respectively. This corresponds to the results reported by Im *et al.* (2011), the stimulation of the P450 2B4 activity by increased concentration of cytochrome b₅ was observed, demonstrating the full biological functionality of the nanoprobe.

The activity experiments in the above mentioned system underlines the advantages of photo-initiated cross-linking compare to the chemical cross-linking (Sulc *et al.* 2012). The conditions used previously for chemical cross-linking experiments dictated by optimal pH value for EDC chemistry (1-ethyl-3-[3-dimethyl(aminopropyl)]carbodiimide hydrochloride, pyridine buffer, pH 6.5) decreased the enzyme activity to 67% of P450 2B4 control activity due to presence of pyridine buffer or lower pH value (Sulc *et al.* 2012). Another advantage of the photo-initiated cross-linking in addition to its compatibility with physiological conditions consists in shorter total time of the cross-linking reaction (in range of minutes instead of hours). Finally, an important advantage of the photo-initiated cross-linking is the unselective reactivity of the highly reactive carbene biradical and the resulting non-specific covalent bond cross-linking. In contrast, for chemical cross-linkers the formation of a new bond is directed by the chemically determined specificity of the used cross-linker (e.g. EDC forms neutral linkage between amino and carboxylic groups). Both these features make it possible to use the photo-initiated cross-linking even for fixation of the non-covalent transient protein-protein complexes within the membrane of the MFO system.

The photolysis of pre-incubated vesicles containing P450 2B4 enzyme with cytochrome b₅ protein nanoprobe produced a mixture of covalently cross-linked

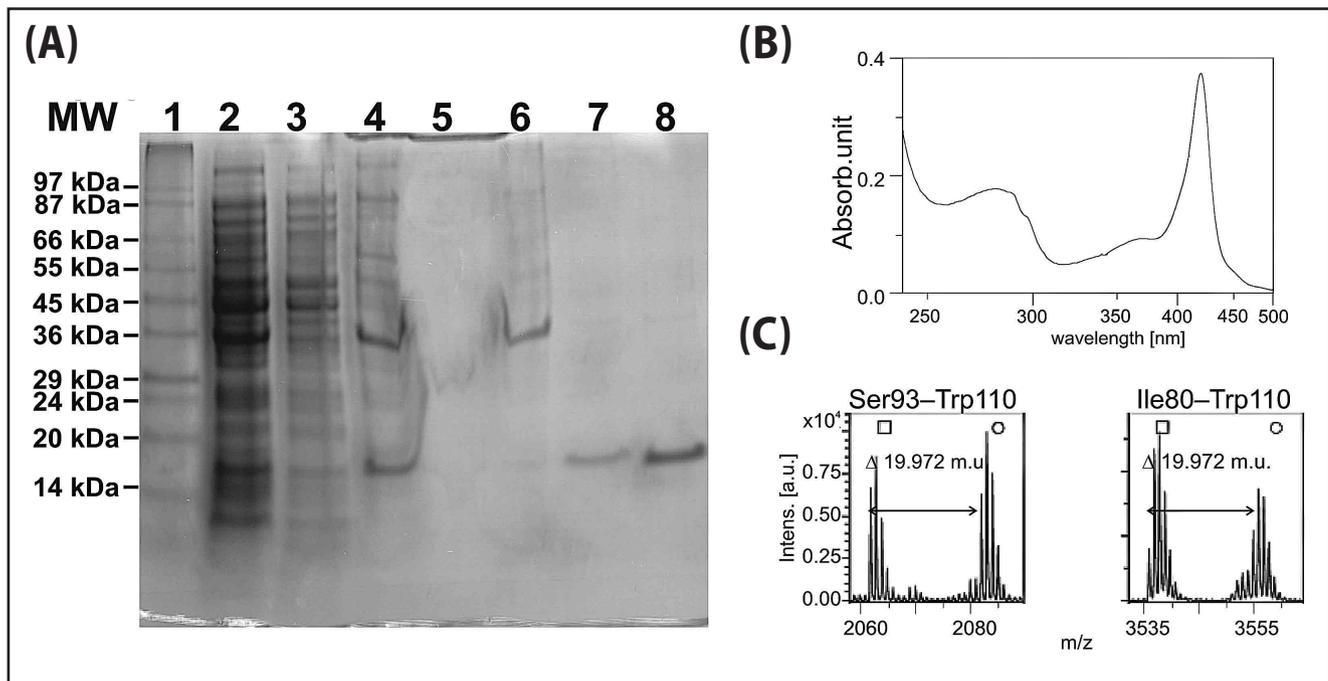


Fig. 1. The characterization of the cytochrome b_5 photo-initiated cross-linking nanoprobe using: (A) 1D SDS-PAGE electrophoresis (15%, CBB R-250 staining, Sigma wide-range molecular weight standards in line 1) – purification process is illustrated in lines 2–8 (2 – *E. coli* lysate, 3 – supernatant after $100\,000 \times g$ ultracentrifugation, 4 – pellet after same ultracentrifugation, 5 – supernatant after protein solubilisation with detergent, 6 – pellet from the same sample like in line 5, 7 – collected fractions after the first DEAE-Sepharose CL4B, 8 – final preparation), (B) UV-VIS spectrum of 20 times diluted final preparation in range 240–500 nm loaded in cm^{-1} mode, or (C) MALDI-TOF MS of digested protein band from 1D SDS-PAGE using chymotrypsin (the selected m/z signals 2081.98 or 3256.61 correspond to peptides containing the Met96, Ser93-Trp110 or Ile80-Trp110, respectively. A circle labels the signal of peptide with Met and a square marks corresponding signal of same peptide with incorporated pMet with mass difference – 19.972 m.u.).

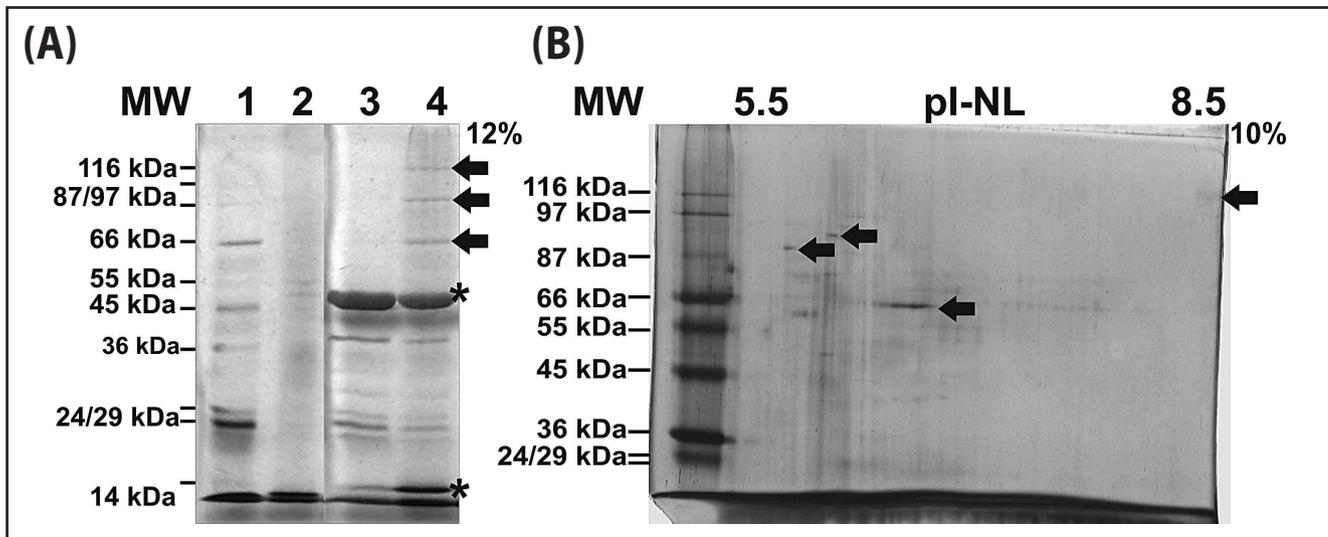


Fig. 2. Monitoring of the cytochrome b_5 :P450 2B4 heterooligomers formation using photo-initiated cross-linking on (A) 12% 1D SDS-PAGE electrophoresis (silver staining, Sigma wide-range molecular weight standards). Arrow labels the heterooligomeric product and asterisk marks both monomers (cytochrome b_5 theoretical MW of 15 kDa and P450 2B4 theoretical MW of 56 kDa), or (B) 10% 2D SDS-PAGE electrophoresis (pI range from 5.5 to 8.5, silver staining, Sigma wide-range molecular weight standards). Arrow labels the heterooligomeric product; both monomers are not present (out of pI range of used IPG strip).

oligomers. It most probably intercept the distribution of their different ratios and novel covalent junctions between both proteins. The proportion of the orientations statistically depends on the presence of each species in the reaction mixture. The electrophoresis (1D

SDS-PAGE) of the photolytic product has shown, in addition to the bands of both monomers (cytochrome b_5 and P450 2B4), three newly formed protein bands with lower mobility (see Figure 2A). The peptide mass fingerprinting approach revealed the presence of m/z

values corresponding to peptides generated by trypsin digestion from both proteins in all three bands (data not shown), thus confirming that they correspond to heteromeric complexes.

However, the question remains concerning the molar ratio of both proteins in each of the cross-linked species to suppose the situation how the both enzymes of the MFO system might be organized in the membrane in organisms.

As the first clue, we may use the deduced molecular weights (MW) of each protein band detected using 1D SDS-PAGE which are 68 ± 5 kDa, 88 ± 5 kDa and 125 ± 5 kDa. We may calculate the expected values for observed complexes on the basis of MW of individual constituents – 15 kDa for cytochrome b₅ and 56 kDa for P450 2B4. The first and the second observed value can be thus easily and rather unequivocally interpreted as

P450 2B4:cytochrome b₅ complexes with molar ratios of 1:1 (expected MW of 71 kDa) and 1:2 (expected MW of 86 kDa), respectively. Alas, the third deduced MW value of 125 kDa cannot be easily interpreted. It can correspond to several different heterooligomers; the P450 2B4:cytochrome b₅ complex with molar ratio 2:1 (expected MW of 127 kDa), 1:4 (theoretical MW of 117 kDa), or perhaps even 1:5 (theoretical MW of 132 kDa). However, we cannot completely exclude the theoretical possible complex with molar ratio 1:3 (expected MW of 102 kDa), because the cross-linked species may display aberrant mobility in 1D SDS-PAGE.

Therefore, to distinguish between the possible complexes discussed above, 2D SDS-PAGE was employed (see Figure 2B). This method allows to determine not only MW of protein band, but also the value of its isoelectric point (pI). These isoelectric points should differ for each complex, reflecting their different composition. The theoretical values of MW and pI were calculated using ExPASy tools Compute pI/Mw (Bjellqvist *et al.* 1993). The P450 2B4:cytochrome b₅ complex with molar ratio 2:1 has the theoretical pI 8.19, the complex with molar ratio 1:3 the theoretical pI 5.94, for complex 1:4 the value of theoretical pI is 5.79, and for molar ratio of 1:5 the pI value is theoretically 5.69. Although the 4 times higher amount of cross-linked reaction was used for 2D SDS-PAGE than was applied at 1D SDS-PAGE, only silver staining was able to visualize four protein spots of the oligomeric complexes (see arrows in Figure 2B). The loss of material was probably caused during acetonitrile precipitation or insufficient adsorption of sample into IPG. Unfortunately, the higher amount of cross-linking mixture used for acetonitrile precipitation revealed very low resolution of electro-focusing process at 2D SDS-PAGE, and therefore could not be used.

Examining the results of 2D SDS-PAGE depicted at Figure 2B, we should start with the fact that both monomers are not visible in the gel, because their pI values are out of the separation range (P450 2B4 theoretical pI 9.01, cytochrome b₅ theoretical pI 5.14). The most intense broad band with MW corresponding to 68 ± 5 kDa has estimated pI $6.5-6.7\pm 0.5$, confirming its P450 2B4:cytochrome b₅ molar ratio 1:1 (theoretical pI 6.74). The two additional spots with similar MW (90 ± 5 kDa and 93 ± 5 kDa) correspond to the second band in 1D SDS-PAGE and have estimated pI values of 5.9 ± 0.5 and 6.1 ± 0.5 , respectively. Therefore, these two species probably may be ascribed to different orientations of the covalent complex containing P450 2B4:cytochrome b₅ in the molar ratio 1:2 (theoretical pI 6.18, MW 86 kDa). The small apparent differences are likely to reflect the aberrant behavior of large complexes in SDS electrophoresis. The proteolysis of these three cross-linked species to peptides using trypsin and the following MALDI-TOF MS analysis of the resulting peptide mixtures confirmed the presence of both P450 2B4 and cytochrome b₅ (detected *m/z* values corresponding to peptides generated by trypsin from both

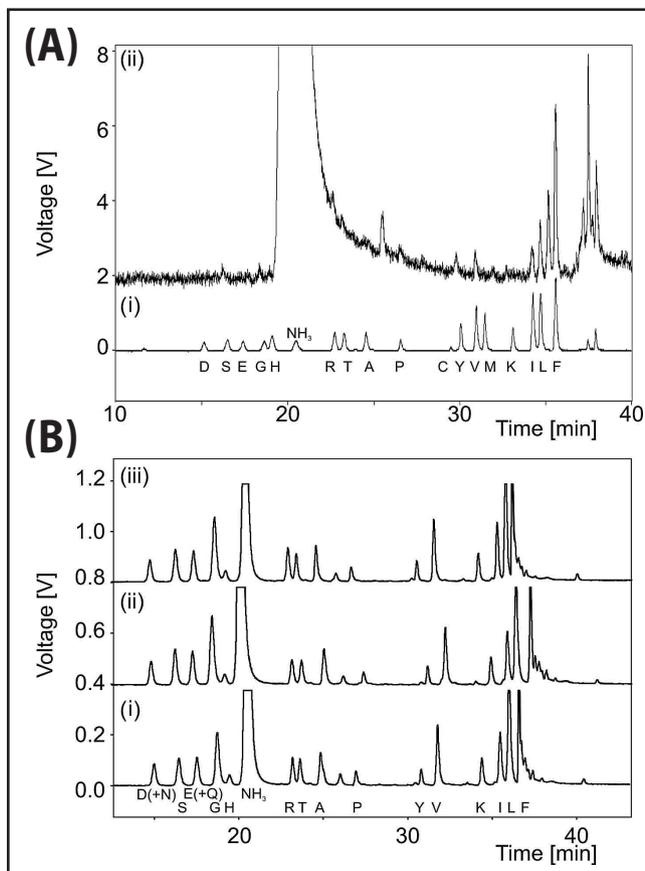


Fig. 3. The HPLC chromatogram displaying the quantities of modified amino acid. (A) The separation of (i) equimolar standard mixture of amino acids and (ii) directly proteolyzed protein band (cytochrome b₅:P450 2B4 heterodimer in 1:1 molar ratio) excised from 12% SDS-PAGE polyacrylamide gel (the HPLC chromatogram is shifted 2 V units to the top). (B) The separation of mixtures of amino acids from protein bands corresponding to heterooligomeric cytochrome b₅:P450 2B4 complexes electroblotted at a PVDF after 1D SDS-PAGE electrophoresis: protein band with MW of (i) 68 ± 5 kDa, (ii) 88 ± 5 kDa, and (iii) 125 ± 5 kDa (the HPLC chromatograms marked (ii) and (iii) are shifted 0.4 V or 0.8 V units to the top, respectively). The one letter amino acid abbreviation or NH₃ for ammonia labels corresponding elution peak (below each peak) on the bottom of each picture.

proteins, data not shown). Unfortunately, the last spot (corresponding to the highest molecular weight band in 1D electrophoresis) has very low intensity. Nonetheless, the estimated pI value of $7.9\text{--}8.0\pm 0.5$ and MW of 125 ± 5 kDa match the values for the complex with P450 2B4:cytochrome b_5 molar ratio 2:1 (theoretical pI 8.19 and MW 127 kDa), but the lower quality of these data with very low intensity and quality of corresponding MS signals (no detectable m/z signal of peptide generated from both protein) dictates certain restraint.

To quantify the composition of oligomeric P450 2B4:cytochrome b_5 covalent complexes, the determination of amino acid in one cycle during the *N*-terminal Edman analysis was employed. Although the 8 times higher amount of cross-linking reaction mixture (compared to 1D-SDS-PAGE) was used for the electroblotted protein bands with following Edman analysis, no positive result was obtained for any of the analyzed species as the analyzed amounts were under sensitivity limits of this methodology approach. To increase intensity of signal and overcome the low detection limit of *N*-terminal Edman degradation (only the amount of one amino acid corresponding to a molar amount of total protein is detected during one cycle), total hydrolysis of protein bands separated by 1D SDS-PAGE with following amino acid modification and HPLC separation was employed. As it is shown in Figure 3A(i), almost all amino acid species from the used standard mixture were separated with base-line resolution. The proteolysis of polyacrylamide gel pieces containing protein by hydrochloric acid resulted in a production of a high amount of ammonia from polyacrylamide that is in extreme over-abundance to amino acids of protein sample and therefore interfered during separation and dramatically decrease the sensitivity of applied HPLC method. To eliminate this interference, the total proteolysis of electro-blotted protein band at a PVDF membrane was used and HPLC chromatograms of all three oligomeric complexes are shown in Figure 3B [species having MW of (i) 68 ± 5 kDa, (ii) 88 ± 5 kDa, and (iii) 125 ± 5 kDa]. In each analyzed protein band a total area under curve (AUC) was determined for each amino acid. Every value of AUC was normalized using calculated number of particular residues of corresponding amino acid present in proposed specie. To calibrate the method for each individual amino acid, the standardization of its normalized AUC area in complex using determined normalized AUC area for the same amino acid in one of the analyzed monomer with known number of amino acid residues (*X* for cytochrome b_5 or *Z* for P450 2B4) was performed. The value of this standardized ratio determined by this method should be stable for any heterooligomeric complex properly assessed stoichiometry of two proteins with known sequence (amino acid composition). As shown in Table 1, only expecting the molar ratio of P450 2B4:cytochrome b_5 2:1, we obtained the correct values for both calibrations, *X* or *Z* values, for all tested amino acids. This observa-

tion significantly supports the proposed composition of the P450 2B4:cytochrome b_5 heterooligomeric complex with MW of 125 ± 5 kDa, establishing its molar ratio as 2:1. Other proposed compositions significantly differ in one or more determined values of *X* or *Z* (shown as bold numbers in Table 1) and thus might be excluded.

The question, however, is how to interpret the obtained results. Can we extract any information from heterooligomer complex formation? Although much information was previously described, the structural details and functional relevance of the P450-membrane and a mutual protein-protein interaction within the MFO system are not fully understood. It is well known that both cytochromes are tightly anchored in the membrane bilayer by a single transmembrane helix (the first 66 amino acids from *N*-termini together with this *N*-terminal alpha helix of the hydrophobic F-G loop for P450 (Nelson & Strobel 1988, Pernecky *et al.* 1993), and cytochrome b_5 C-terminal transmembrane domain that contains two methionine residues at position 126 and 131. Recently, the distortion of transmembrane orientation for both proteins was determined using solid-state NMR (for P450 17° and for cytochrome b_5 14° tilt from the lipid bilayer normal) (Yamamoto *et al.* 2013a). This tilted orientation of both transmembrane domains in the lipid bilayer probably allows the interaction between them neither significantly altered the helical structure of the transmembrane domain of cytochrome b_5 nor does it alter its geometry. This interaction and potential co-operativity was also support by different observation of the motion restriction (Yamamoto *et al.* 2013b, Stier *et al.* 1991). Therefore, for the MFO system some sort of molecular organization and regulation, if it is to function properly, could be proposed.

Taking all our data together, the presented results demonstrate the capability of the photo-initiated cross-linking approach to study interactions of proteins within membrane environment. The used methodology reveals the formation of not only the P450 and cytochrome b_5 heterodimeric complex, but also the multimeric complexes within the membrane. These multimeres preserve the symmetric total P450:cytochrome b_5 molar ratio of 1:1 and probably at least two mutual orientations could be suggested (existence both heterooligomeric species P450:cytochrome b_5 , the molar ratio 1:2 and 2:1, were determined at 1D SDS-PAGE). The similar protein band pattern was also detected using chemical cross-linking that in combination with MS analysis revealed two mutual orientations between P450 and cytochrome b_5 (Sulc *et al.* 2012). Therefore, the formation of such multimeric structures could be also expected within the MFO system located in the endoplasmic membrane bilayer. The prepared cytochrome b_5 nanoprobe combined with photo-initiated cross-linking should be a challenging tool to study such structures not only in the reconstituted artificial proteo-liposomes as shown here, but also in the natural liver microsomal membranes.

Tab. 1. The values of determined ratios of the P450 2B4:cytochrome b₅ complexes after processing of AUC HPLC amino acid analysis data (area under curve).

Determined aminoacid	Value of	Complex P450:cytochrome b ₅ in molar ratio						Average	S.D.
		1:1	1:2	2:1	1:3	1:4	1:5		
R	theor. q.	42	46	80	50	54	58		
	X	0.21	0.22	0.25	0.4	0.37	0.35	0.23	0.02
	Z	1.4	1.42	1.64	2.63	2.43	2.26	1.49	0.13
V	theor. q.	37	44	67	51	58	65		
	X	0.31	0.28	0.31	0.41	0.35	0.32	0.30	0.02
	Z	1.69	1.46	1.64	2.16	1.89	1.69	1.60	0.12
L	theor. q.	77	90	141	103	116	129		
	X	0.37	0.35	0.34	0.46	0.41	0.37	0.35	0.02
	Z	1.16	1.11	1.06	1.45	1.29	1.16	1.11	0.05
E (+Q)	theor. q.	58	72	102	86	90	104		
	X	0.42	0.44	0.41	0.49	0.47	0.41	0.42	0.02
	Z	1.27	1.3	1.23	1.46	1.39	1.21	1.27	0.04
G	theor. q.	41	47	76	53	59	65		
	X	0.33	0.34	0.29	0.42	0.37	0.34	0.32	0.03
	Z	1.53	1.59	1.4	1.86	1.67	1.52	1.51	0.12
F.	theor. q.	44	47	85	50	53	56		
	X	0.19	0.21	0.17	0.28	0.27	0.25	0.19	0.02
	Z	1.11	1.19	0.96	1.61	1.53	1.44	1.09	0.12
I	theor. q.	35	43	62	51	59	67		
	X	0.58	0.56	0.54	0.64	0.56	0.49	0.56	0.02
	Z	1.26	1.2	1.14	1.39	1.20	1.06	1.20	0.06
T	theor. q.	36	46	62	56	66	76		
	X	0.47	0.48	0.38	0.42	0.36	0.31	0.44	0.06
	Z	1.26	0.91	1.03	1.14	0.97	0.84	1.07	0.18
D (+N)	theor. q.	54	69	93	84	99	114		
	X	0.4	0.47	0.42	0.47	0.40	0.35	0.43	0.04
	Z	1.05	1.23	1.11	1.23	1.04	0.90	1.13	0.09
K	theor. q.	30	40	50	50	60	70		
	X	0.55	0.52	0.57	0.57	0.83	0.71	0.55	0.03
	Z	0.88	0.87	0.92	0.92	1.31	1.15	0.89	0.03

Legend: single letter amino acid abbreviations are used for each amino acid {aa} and its theoretical quantity (theor.q.) in protein band is shown, in both values X and Z the area under curve (corresponding to chromatographic peak) of each determined amino acid is used, the used formulas for calculation of values X and Z are $X = \text{area}\{\text{aa}\}\text{complex} / [\text{theor.q.}\{\text{aa}\}\text{complex} * (\text{area}\{\text{aa}\}b_5 / \text{theor.q.}\{\text{aa}\}b_5)]$, $Z = \text{area}\{\text{aa}\}\text{complex} / [\text{theor.q.}\{\text{aa}\}\text{complex} * (\text{area}\{\text{aa}\}P450 / \text{theor.q.}\{\text{aa}\}P450)]$, average and S.D. is calculated from three values of P450:cytochrome b₅ complexes in molar ratio 1:1, 1:2, and 2:1, bolded number of X or Z values marks significant difference from its proper value.

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