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Cytochrom P-450: studium struktury a interakcí metodami chemické modifikace, foto-iniciovaného síťování a hmotnostní spektrometrie

Cytochrome P-450: Study of structure and interactions using chemical modification, photo-initiated cross-linking and mass spectrometry

PhD thesis

Supervisor: Assoc. Prof. RNDr. Miroslav Šulc, PhD.

Prague, 2015

Dedicated to my family,
who supported me throughout my studies.

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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ABSTRAKT

Systém oxygenas se smíšenou funkcí se v organismu podílí na biosyntéze endogenních a také metabolismu exogenních látek (např. léčiv nebo chemických prokarcinogenů). Substráty jsou biotransformovány terminálními oxygenasami systému – cytochromy P450 (P450). Katalytické vlastnosti některých jejich zástupců (např. studované izoformy 2B4) jsou pozměněny v přítomnosti redoxního partnera – cytochromu b_5 (cyb5). Oba cytochromy jsou ukotveny hydrofobními doménami v lipidické membráně endoplasmatického retikula, zatímco jejich katalytické domény jsou exponovány do cytosolu buňky.

K rozšíření současných znalostí o struktuře a interakcích studovaných cytochromů byly využity dva přístupy založené na kovalentním síťování aminokyselin v „nulové vzdálenosti“ (angl. „zero-length cross-linking“): (1) chemické síťování činidlem 1-ethyl-3-(3-dimethylaminopropyl)carbodiimid (EDC) propojujícím ve vodném prostředí přístupné karboxylové skupiny s primárními aminy, a (2) síťování pomocí foto-labilního analogu methioninu (pMet), který se po aktivaci UV zářením může vázat na libovolnou aminokyselinu, a to v hydrofobním i hydrofilním prostředí. pMet byl do sekvence cyb5 inkorporován namísto methioninu během rekombinantní exprese proteinu v *E. coli*, která probíhala v limitním médiu obohaceném o aminokyselinový analog. Optimalizací experimentálních podmínek bylo dosaženo přibližně 20-30% substituce přirozené aminokyseliny.

Zesítěné heteromery byly separovány na 1-dimenzionální elektroforéze, a komplexy o nejednoznačně určené stechiometrii byly charakterizovány pomocí 2-dimenzionální elektroforézy a metodou celkové aminokyselinové analýzy. Ve směsi získané proteolýzou jednotlivých oligomerů P450 2B4:cyb5 (1:1, 1:2 nebo 2:1) byly zesítěné peptidy identifikovány pomocí hmotnostní spektrometrie s vysokým rozlišením ve spojení s kapalinovou chromatografií.

Foto-síťování vůbec poprvé přímo potvrdilo interakci hydrofobních helixů cyb5 a P450 2B4 v prostředí lipidické membrány, a ukázalo také doposud nepopsané kontaktní oblasti obou proteinů v cytosolu. Další aminokyselinové páry podílející se na kontaktu katalytických domén byly zachyceny pomocí činidla EDC, a výsledky byly využity při *in silico* modelování této interakce. Prezentovaná zjištění podporují obecně přijímanou topologii obou cytochromů při níž dochází k přenosu elektronu. Navíc však naznačují další možné natočení proteinů, které je pro přenos elektronu nevhodné, může však být zodpovědné za alosterickou modulaci P450 2B4. Existenci minimálně dvou orientací potvrzuje i vznik heterotrimerních komplexů se stechiometrií cytochromů 1:2 a 2:1.

Výsledky demonstrují výhody nově vyvinutého foto-iniciovaného síťování pro studium transientních protein-proteinových interakcí oproti síťování chemickému: (1) vazba pMet na jakoukoliv aminokyselinu v blízkosti (2) nezávisle na okolním prostředí (membrána, cytosol), (3) úspěšné zavedení foto-aktivovatelné aminonyseliny na požadovaná místa sekvence proteinu pomocí místně cílené mutageneze, a (4) vysoká rychlost reakce vzniklých biradikálů, které zachycují okamžité uspořádání systému, včetně více souběžně interagujících proteinů.

ABSTRACT

Mixed function oxygenase system participates in biosynthesis of endogenous and metabolism of exogenous substances (*e.g.* drugs or chemical procarcinogens) in an organism. Substrates are biotransformed by terminal oxygenases – cytochromes P450 (P450). Catalytic properties of certain P450s (*e.g.* studied isoform 2B4) are altered in the presence of a redox partner – cytochrome b_5 (cyb5). Both cytochromes are anchored by hydrophobic domains in a lipid membrane of endoplasmic reticulum whereas their catalytic domains are exposed to cytosol.

Two zero-length cross-linking approaches were employed to extend present knowledge of P450 2B4 and cyb5 protein structure and protein-protein interactions: (1) interlinking of carboxylate and primary amine groups of amino acids by water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and (2) photo-initiated cross-linking by photo-labile methionine analog (pMet), which links to any amino acid after activation by UV-irradiation, either in hydrophilic or hydrophobic environment. pMet was incorporated to methionine site(s) of cyb5 during recombinant expression in *E. coli*, which was carried out in limit medium supplemented with amino acid analog. Optimization of experimental conditions led to ~20-30% substitution of the natural amino acid.

Covalent complexes of various stoichiometries arisen from cross-linking were separated by 1-dimensional electrophoresis, but the molecular weigth and consequently the protein ratio of the heteromers had not been deduced conclusively. Therefore 2-dimensional electrophoresis and total amino acid analysis were employed to determine P450 2B4:cyb5 ratios (1:1, 1:2 or 2:1). Individual assemblies were proteolytically digested and the cross-links were identified in the resulting peptide mixture by high resolution mass spectrometry coupled to liquid chromatography.

Photo-initiated cross-linking directly identified interaction of cyb5 and P450 2B4 hydrophobic helices in the lipid membrane environment for the first time, and also revealed yet unknown contact regions of both proteins in cytosol. More amino acids of the catalytic domains were fixated by EDC agent, and the acquired data served as a basis for *in silico* modeling of this interaction. Presented findings support generally adopted topology of the cytochromes, which is suitable for electron transfer. Additionally, they also indicate distinct protein orientation, which is improper for the electron donation, however could be responsible for allosteric modulation of P450 2B4. Also the formation of heterotrimeric complexes with cytochrome stoichiometries 1:2 and 2:1 validates the existence of at least two mutual protein orientations.

The results demonstrate advantages of novel photo-induced cross-linking in comparison to conventional chemical cross-linking for transient protein-protein interactions determination: (1) the binding of pMet to any amino acid side chain in its close proximity (2) independently on surrounding environment (cytosol, lipid membrane), (3) successful introduction of photo-reactive amino acid analogue to the requested sites in the sequence by site directed mutagenesis, and (4) the rapid reaction of carbene biradicals capturing momentary organization of the system, including more concurrently interacting proteins.

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ACRONYMS

Å	Angstrom
aaRS	Aminoacyl-tRNA synthetase
AUC	Area under curve
cyb5	Cytochrome b_5
DLPC	Dilauroylphosphatidylcholine
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EM	Electron microscopy
ESI	Electrospray ionization
FT-ICR	Fourier transform ion cyclotron resonance
K_D	Dissociation constant
kDa	Kilodalton
LC	Liquid chromatography
m/z	Mass-to-charge
MALDI	Matrix-assisted laser desorption/ionization
MAS	Magic angle spinning
Met	Methionine
MFO	Mixed function oxidase
mg/l	Milligram per liter
ml	Milliliter
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NADH	Reduced form of nicotinamide adenine dinucleotide
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
nm	Nanometer
NMR	Nuclear magnetic resonance
P450	Cytochrome P450
pI	Isoelectric point
pMet	Photo-methionine (L-2-amino-5,5'-azi-hexanoic acid)
ppm	Part per million
PVDF	Polyvinylidene fluoride

SDM	Site-directed mutagenesis
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate
TOF	Time-of-flight
tRNA	Transfer ribonucleic acid
TROSY	Transverse relaxation optimized spectroscopy
UV	Ultraviolet
WT	Wild type

1. INTRODUCTION AND METHODS

Macromolecules (proteins, nucleic acids, carbohydrates, and lipids) carry out the most functions in a living cell. Structural biology is a discipline which looks into their spatial arrangement in an effort to understand in detail (1) what physiological role(s) do they fulfill, (2) how do they affect each other, and (3) the way they are compiled together in functional complexes. Structural findings contribute to *in silico* model building, working out the mechanism of their action and help predict behavior and function of yet unexplored macromolecules. Rapid development in this research field is substantially facilitated by (i) genome sequencing, which provides templates for recombinant expression (International Human Genome Sequencing Consortium 2001), (ii) well-established hosts producing sufficient amounts of the protein (Rai and Padh 2001), and (iii) advanced instrumentation and high-performance computers for data acquisition, processing and evaluation. The features of dominant, mutually complementing approaches providing insight into the tertiary and quaternary structure of proteins with special emphasis on the techniques used within the bounds of this thesis are content of following chapters.

1.1. Methods for protein structure determination

X-ray crystallography is contemporarily the leading method for structure determination, which gives rise to the most protein models. It owes this primacy in large extent to considerable amount of automation in the course of the whole process – crystallization, data collection, structure assignment, and model building (Egli 2010). Nowadays, high-intensity X-rays originating in synchrotrons promise atomic-resolution structural data (one of the most detailed crystal structures with 0.48 Å resolution is crambin protein (Schmidt *et al.* 2010)) (Dauter *et al.* 2010). Protein crystals elastically diffract high-energy photons whose angles and intensities are subsequently recorded. Two parameters – amplitude and phase – have to be assessed to reconstruct the 3-dimensional electron density map into which the individual atoms are assigned; the former can be calculated directly from the acquired data while the latter is systematically lost in a measurement (Egli 2010). Multi-wavelength anomalous diffraction technique, which investigates crystal containing anomalously diffracting atoms (most often selenium) at different wavelengths, is one of the ways to assess the phase (Karle 1980; Hendrickson *et al.* 1985). For this purpose, proteins with selenomethionine instead of methionine (Met) in

the sequence are recombinantly expressed (Hendrickson *et al.* 1990). The same principle uses also the methodology based on photo-initiated cross-linking, and will be mentioned later on. The model based on the initial phases is further refined by molecular modeling techniques and more accurate phases are determined. However even at this point, the model neither corresponds with the protein structure in solution nor reports on the protein dynamics (Egli 2010). Furthermore, the highly flexible or disordered proteins as well as those that require hydrophobic environment to maintain their fold are rather reluctant to crystallize, and can exceed the capabilities of this technique. Hydrophobic domains of membrane proteins can be truncated by a protease and only solvent exposed domain can be crystallized to circumvent the obstacle, as was shown in case of cytochrome P450 2B4 cytosolar domain structure sloved at 1.6 Å resolution (Scott *et al.* 2003).

Electron microscopy (EM) is a diffraction technique, which instead of X-rays observes scattering of electrons by atoms. Electrons interact with protein molecules more strongly, therefore only thin sections of specimens (~0.1 micrometer) instead of a crystal (minimally 0.1 millimeter) are analyzed. In contrary to X-ray crystallography, the phase information necessary for the object reconstruction is established directly from the acquired data (Erickson and Klug 1971). However, the protein structure resolution achieved by EM is only near-atomic, as demonstrates the ribosome subunit structure solved at 3.6 Å resolution (Greber *et al.* 2014). The technique is predominantly used for visualizing contours of large protein complexes and the interactions within them. An example of its application is the study showing cytochromes P450 forming clusters instead of being evenly distributed in microsomes (Matsuura *et al.* 1978). Further, the derived technique called **electron crystallography** can be applied for studying membrane proteins anchored in the lipid bilayer, and like this forming 2-dimensional crystal. This approach was used for solving the structure of aquaporin-0 at 1.9 Å resolution (Gonen *et al.* 2005). However, both electron crystallography and EM are susceptible to introduction artifacts resulting from sample preparation procedures. Therefore, the obtained data have to be critically assessed.

Nuclear magnetic resonance (NMR) is another alternative to probe protein structure, protein-protein and protein-ligand interactions. The behavior of atom nuclei with nonzero spin (most commonly ^{1}H, ^{2}D, ^{13}C, and ^{15}N) differs by the effect of the surrounding atoms in strong magnetic field (Lian and Roberts 2011). The radio frequency pulse sequences providing information about chemically linked atoms, their distances, and speed

of movement in respect to each other in systems up to 50 kDa were developed as an extension of NMR experiments designed originally for analysis of small molecules in the late 1980s, and are plentifully utilized up to the present (Aue at al. 1976). At the turn of the 20th century, transverse relaxation optimized spectroscopy (TROSY) turned up pushing efficient use of *de novo* protein structure determination up to 1 MDa, which brought NMR closer to be fully-fledged alternative to X-ray crystallography (Fiaux *et al.* 2002). In comparison with solution NMR experiments, where the anisotropic interactions between atom nuclei in the magnetic field are averaged due to Brownian motion of molecules, limited movement of membrane bound proteins prevents anisotropy averaging (Hong *et al.* 2012). To circumvent related spectral line width broadening (decreasing resolution) the solid-state NMR experiments were developed, most often utilizing magic angle spinning (MAS) – spinning the sample at the angle 54.74° with respect to the direction of magnetic field, which leads to narrower spectral lines (and thus higher resolution) (Andrew *et al.* 1958). As an example, the structure of cytochrome b_5 was solved by the combination of TROSY experiment in solution NMR (high resolution structure of soluble cytosolic domain) and MAS experiment in solid-state NMR (topology of hydrophobic membrane anchor) (Ahuja *et al.* 2013). However, further investigation of the membrane domain structure is required to supplement contemporary low resolution data.

In contrary to X-ray crystallography and solid state NMR, the solution NMR experiments are performed in aqueous buffers, which better resembles native conditions. Moreover, protein dynamics can be deduced from the resulting data. However, the necessity of 0.05 to >1 millimolar protein concentration for the measurement can also introduce nonbiological intermolecular interactions altering *in vivo* conformation (Lian and Roberts 2011).

Alternative approaches combine complementary methodologies, which provide comprehensive, more reliable description of a protein structure, and elucidate how proteins interact with their binding partners, substrates and ligands. Frequently, **chemical modification** and **cross-linking experiments** followed by highly accurate and sensitive mass spectrometric analysis identifying interaction sites are employed to gather structural information and to confirm proposed mechanism of protein actions. The variety of available cross-linking agents possessing diverse features offers the possibility to perform a broad range of experiments that have several advantages in common: (1) they are performed under the (near) physiological conditions, (2) the analysis is fast and require

trace amounts of sample, (3) the molecular weight of studied proteins or complexes is theoretically unlimited, and (4) both flexible and membrane regions can be addressed. Additionally, novel modification and cross-linking agents are being developed, following the aim of solving protein structure entirely by means of these techniques.

Computational methods are indispensable part of protein structure determination process. They not only deal with the protein sequence analysis, *ab initio* structure predictions and visualization of molecules but also perform calculations based on experimental data, minimize potential energies of the structures and simulate molecular dynamics to make the models more realistic and accurate.

1.2. Mass spectrometry in proteomics

Mass spectrometry (MS) is a versatile technique for fast and sensitive identification of chemical species according to their mass-to-charge (*m/z*) ratio. The measurements are carried out in a mass spectrometer which consists of 3 substantial elements. First, the analyzed sample is introduced into an **ion source**, where the electroneutral molecules of the analyte are volatilized and charged, the positive or negative ions are introduced into an evacuated **analyzer**, where they are separated owing to their different trajectories in electric or magnetic field according to their *m/z* ratio, and finally they are recorded using a **detector**.

1.2.1. Soft ionization techniques

The substantial expansion of MS to the biological field was enabled in 1980s by the discovery of two soft ionization techniques capable of ionization of macromolecules without distinct fragmentation. The significance of both breakthrough techniques was acknowledged by awarding K. Tanaka and J. Fenn Nobel prize in 2002.

Matrix-assisted laser desorption/ionization (MALDI) (Tanaka *et al.* 1988) is based on crystallizing biomolecules with the great excess (1:10,000) of low molecular weight matrix molecules eligible to absorb the energy of laser pulse. Weak organic acids containing conjugated double bond system are usually employed (*e.g.* α-Cyano-4-hydroxycinnamic acid or 2,5-dihydroxy benzoic acid for peptide analysis). Matrix is (de)protonized and through its vaporization the biomolecules are carried out into the high vacuum; proton transfer is arranged during mutual collisions in a gas phase and consequently leads to ionization of peptides/proteins of interest, which are further

analyzed. The macromolecules become mainly singly charged, which results in easily interpretable spectra, where *m/z* signal matches with (de)protonized molecular weight of the ion. Typically, MALDI is coupled with either "time of flight" (TOF) or "quadrupole time of flight" (qTOF) analyzer, both routinely used for their speed, high sensitivity (can detect as little as attomoles of a short peptide) and theoretically unlimited mass range (Vorm *et al.* 1994).

Electrospray ionization (ESI) (Wong *et al.* 1988) applies high voltage (3-5 kilovolts) to a liquid sample at the end of a heated capillary, which results in its coulomb explosion (dispersion of charged solvent droplets containing analyte). The solvent is evaporated *e.g.* in the steam of inert gas and like so the charge is transferred to the analyzed molecules before they enter evacuated analyzer. Resulting ions have typically multiple charges. On the one hand it dramatically extends mass range of used analyzer. On the other hand it provides hard-to-interpret spectra with overlapping peaks for complex samples, as the molecule in the different charged states is represented by multiple signals. The separation on liquid chromatography (LC) included prior the ionization step considerably simplifies the acquired data (Aebersold and Mann 2003). The recorded spectra are highly reproducible, and can be used for quantitative measurements in contrast with MALDI, where both characteristics are impaired by uneven crystallization of the matrix (Cohen and Chait 1996, Wilm 2011).

1.2.2. Tandem and high resolution mass spectrometry

Two different approaches can be followed to conclusively identify analyzed proteins and peptides depending on available instrumentation.

Tandem MS (MS/MS) (Jennings 1968) can rely on instruments with lower resolution analyzers as it does not depend only on the molecular ion masses, but gathers additional information to confirm their nature. The experiment consists of multiple steps: (1) after ionization, the ions are separated at the first analyzer; (2) the parent ion of interest is selected based on its *m/z*, and transferred to the next section where its fragmentation is processed by one of available methods; (3) resulting daughter ions are monitored by the last analyzer, and a MS/MS spectrum is acquired. The whole multistep experiment could be carried out consecutively in just one compartment at instruments based on a principle of ion trap analyzer; and precursor isolation and its fragmentation can be performed repeatedly (Wong and Cooks 1997). The peptide sequence and position of its

modifications can be derived from spacing between adjacent daughter ions of the same series (Aebersold and Mann 2003).

On the other hand, high resolution analyzers – **Fourier transform ion cyclotron resonance** (FT-ICR) (Wolff and Stephens 1953) or **Orbitrap** (Makarov 2000) – have resolution power in order of millions, which allows separation of isotopical peaks of multiply charged ions arisen from ESI as well as ions differing in *m/z* in the ppm range. With this accuracy even the molecular composition of an ion can be deduced, and thus its identity is either confirmed or excluded. The high resolution of these analyzers also depends on the length of the detection for which undisturbed movement of ions is vital. Thus, the minimal number of collisions with neutral molecules within the analyzer is assured by ultrahigh vacuum (10^{-9} to 10^{-10} millibar) during the measurement (Sciglerova *et al.* 2011).

For analysis, (1) sample ions are introduced into a Penning trap and kept circulating inside by magnetic field; (2) applying oscillating electric field to excitation plates (orthogonal to the magnetic field), ions of the same *m/z* start to circulate in a cluster; (3) clusters of different *m/z* oscillate with diverse frequencies, and induce current in detection plates (orthogonal to the both magnetic field and excitation plates) when passing by them; (4) a complex signal (the sum of all participating frequencies) is converted from time- to frequency-domain by Fourier transformation, and subsequently into a mass spectrum. The MS/MS experiments providing additional structural information about the ions (*e.g.* identification of modifications or cross-link sites) can also be performed in FT-ICR analyzer (Marshall *et al.* 1998).

1.3. Protein cross-linking

Chemical cross-linkers are small molecules containing two or three functional groups capable of forming covalent bond with particular amino acid side chains. Even though large number of reagents has been brought out, they all are based only on several chemistries due to limited number of canonical amino acid functionalities (Wong 1991; Chen *et al.* 2012).

(1) Maleinimide functional group can modify **sulfhydryl group** of cysteine (**Figure 1.1A** on page 18). It is a hard to reach amino acid as it is scarce, and often involved in disulfide bonds or ligand binding (thus unavailable for reaction). On the other hand, introducing cysteine into the protein sequence by site-directed mutagenesis (SDM)

promise good crosslinking results as the reaction is very specific (Gregory 1955; Smyth *et al.* 1964).

(2) Agents containing N-hydroxysuccinimide (or alternatively more soluble sulfo-N-hydroxysuccinimide) and imidoester groups mediate reactions conjugating **amines** (**Figure 1.1B** on page 18) (Bragg and Hou 1975; Lomant and Fairbanks 1976). They are frequent groups especially at the protein surfaces exposed to aqueous environment and are involved in formation of both intarmolecular and intermolecular salt bridges.

(3) Chemistry based on carbodiimides like 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) inserting amide bond between **carboxylate and amine** group is of particular interest for fixation of this kind of transient interactions (**Figure 1.1C** on page 18) (Sheehan *et al.* 1961; Hoare and Koshland 1966).

(4) Making use of emerging **nonselective cross-linkers** containing diazirine, arylazide or benzophenone photo-labile functional group (**Figure 1.1D** on page 18) is contemporarily on the rise. They are inert at the wide range of experimental conditions, and their irradiation with appropriate wavelengths (~250 nm for arylazides, ~360 nm for diazirines and benzophenones) results in highly reactive biradicals capable of modifying any amino acid side chain (Bayley and Knowles 1977). The nonselectivity provides more complex structural information (many different residues are interconnected) but consequently the sensitivity is decreased (not all cross-links are detected).

The heterobifunctional agents containing a sulfhydryl/amine reactive functional group, which initially modifies a target residue, and a photo-labile group, which is activated afterwards and binds unselectively to one of the residues within the cross-liner range, are usually used as a reasonable compromise combining the advantages and minimizing the disadvantages of all above mentioned cross-linker groups.

(5) **Photo-reactive amino acids** – *e.g.* photo-methionine or photo-leucine (**Figure 1.1E** on page 18) – represent a subgroup of nonselective cross-linkers. These photo-labile functional group containing analogs, which are incorporated directly into the protein sequence during its recombinant expression, mimic the natural amino acids. The proteins are subsequently employed as nanoprobes in photo-initiated cross-linking experiment (Suchanek *et al.* 2005; Koberova *et al.* 2013). The photo-reactive amino acid is localized at the fixed position of the protein thus only the other interacting residue needs to be determined, and no agents or solvents, which could alter the protein structure, are added to the reaction mixture.

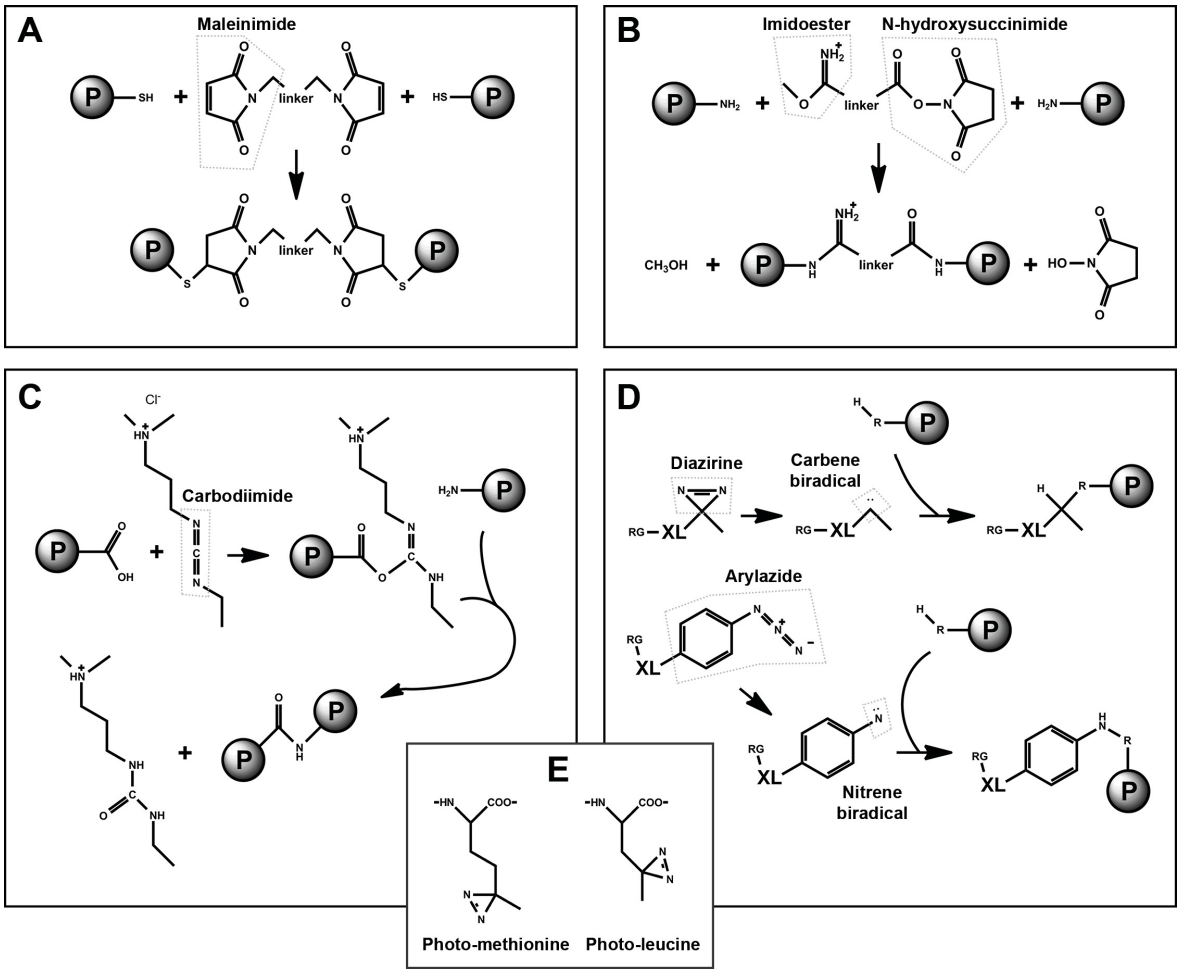

Figure 1.1: Types of cross-linking agents. (A) Homobifunctional cross-linker conjugating sulfhydryl groups, (B) heterobifunctional cross-linker conjugating primary amino groups, (C) zero-length cross-linker conjugating a carboxylate and an amine group, (D) nonselective cross-linkers with a photo-labile functional group, and (E) nonselective photo-reactive amino acid analogs.

1.3.1. Features of cross-linking agents

Suitability of a reagent for a particular experiment derives from its attributes. The **spacer-containing cross-linkers** with variable length serve as molecular rulers, which are convenient for assessing the distance span between amino acids. Moreover, the spacer can contain additional functionalities – affinity tags helping specifically enrich products of the reaction, isotopic tags and/or (within mass spectrometer) cleavable site facilitating identification of cross-links in a mass spectrum (Petrotchenko *et al.* 2010). Also the overall physico-chemical characteristics of the cross-linker can be modulated altering the spacer. However, extra functionalities extend size of the cross-linker, which has impact on its capability to penetrate protein core or to enter active sites and cavities.

On the other hand, the **zero-length cross-linkers** covalently fixate transient intra- and intermolecular interactions within 5 Å distance, without introducing any linker between interconnected functional groups (Kalkhof *et al.* 2005).

The combination of agents bearing various specificities, which would provide sufficient number of unique distance restraints, is necessary to assign the protein structure completely. Unfortunately, contemporary cross-linking agents are not miscellaneous enough and do not provide adequate amount of structural data to achieve this goal yet. Therefore, the most recent effort is focused on developing reagents targeting broader spectrum of amino acids.

1.3.2. Zero-length cross-linkers

The protein-protein interactions in the hydrophilic environment depend greatly on ion pairing of positively and negatively charged amino acid side chains. Well established carbodiimide chemistry (*e.g.* EDC reagent) is routinely employed in structural biology to detect these interactions *e.g.* to probe binding sites for interaction partners on a protein surface. However, not all eligible residues are accessible or available for the reaction and even if so their distribution is uneven and particularly scarce in hydrophobic regions (protein core, transmembrane domain). The chemical reagents also have inherent restrictions like insolubility in aqueous buffers or necessity of certain non-physiological pH for reaction (Wong 1991).

Recently emerged photo-reactive amino acid analogue photo-methionine (pMet) also acts as a zero-length cross-linker (Suchanek *et al.* 2005; Koberova *et al.* 2013). Its activation by UV-irradiation leads to highly reactive biradical with low specificity, which binds indiscriminately to the side chain in its close proximity regardless of experimental conditions and surrounding environment (Bayley and Knowles 1977). pMet substitutes naturally occurring methionine in the protein sequence, and can be additionally introduced to any other desired location of the protein sequence by SDM. However, besides these advantages over chemical cross-linker EDC, radical character of the reaction also entails drawbacks: (1) much larger possible reaction site resulting in several low abundance species, which complicates their detection and identification of exact points of cross-linking, (2) worse ionization of rather hydrophobic linked peptides originating from the protein core or the membrane domain, where methionine residues are often localized, and

(3) extensive computational demands as more theoretical cross-links have to be taken into account.

1.4. Introduction of unnatural amino acids into protein sequence

Translation is a process in which genetic information is converted from a polynucleotide sequence into a polypeptide chain. During the process, a ribosome moves along a mRNA molecule and pairs triplet codons with complementary anticodones of aminoacyl-tRNAs (Crick *et al.* 1961). A unique aminoacyl-tRNA synthetase (aaRS) handles correct coupling of an amino acid with a corresponding tRNA (in a two-step process involving ATP-dependent amino acid activation) (Ibba and Soll 2000). The course of protein synthesis is highly regulated to safeguard the protein sequence fidelity. The aaRSs have highly selective binding sites for both tRNA and appropriate amino acid, and several of them also possessed editing function eliminating mis-acylated tRNAs (Nureki *et al.* 1998; Silvan *et al.* 1999). Nonetheless, certain aaRSs (*e.g.* for methionine) have more relaxed amino acid binding site and allows structurally closely related analogs to conjugate with tRNA (Dezniak and Barciszewski 2001).

Several methods were developed to circumvent natural translation apparatus in order to engineer proteins with new functionalities (photo-induced switching (Bose *et al.* 2006), redox sensitive proteins (Alfonta *et al.* 2003), hyperstability (Tang *et al.* 2001), protease resistance (Meng and Kumar 2007) etc.). The technology is based on incorporation of unnatural amino acids with side chains bearing desired attributes into the protein.

1.4.1. Site-specific incorporation of amino acids

Incorporation of unnatural amino acid to the specific position in the protein sequence is inspired by the representatives of *Methanosarcina* genus. These methane producing *Archaea* have evolved an extra aaRS/tRNA pair for amino acid pyrolysine, in addition to 20 aaRS/tRNA pairs for natural amino acids utilized in proteosynthesis. The aaRS for pyrolysine is orthogonal (non-crossreactive to other amino acids) and corresponding tRNA accommodates anticodon complementary to amber (UAG) stop codon (Hao *et al.* 2002; Srinivasan *et al.* 2002). Similarly, selenocysteine can be planted into a protein at the position of the "opal" (UGA) stop codon (Chambers *et al.* 1986). Whether the non-canonical amino acid is incorporated or protein synthesis is terminated

instead at the dual meaning codon is directed by the presence of a supplementary signal within mRNA molecule or by bare competition between binding of the aminoacyl-tRNA and the release factor (Atkins and Baranov 2007).

The modified orthogonal tRNA/aaRS pair from another species is introduced to the demanded expression host organism to enable the site-specific incorporation of non-canonical amino acid to recombinant proteins. The novel tRNA bears mutation at the anti-codon triplet to complement stop codon sequence; and the respective aaRS carries mutations at the binding site which can consequently accommodate the amino acid analog of interest. The overall efficiency of stop codon "read-through" (and so the yield of amino acid incorporation) ranges from 10 to 40 %, and markedly reduces protein production. Besides, the effect of decreased production is multiplied when the protein consists of more than one stop codon that has to be read-through at the same time (more than one unnatural amino acid is being introduced into a protein). However, almost complete incorporation of amino acid analog is achieved in the final full length protein. Even though the yield of the protein is generally poor (1-10 mg/l), production achieved in optimized expression systems can be considerably enhanced (up to 800 mg/l) (Cho *et al.* 2011).

1.4.2. Residue-specific incorporation of amino acids

The second widely used set of techniques is based on reassignment of a sense codon. As a matter of principle, cells are grown in a medium deficient in one of the natural amino acids, but supplemented with its structurally related analog which is incorporated into the protein sequences globally. Typically (but not inevitably), auxotroph cells unable to synthesize the particular amino acid are utilized for this type of protein expression (Cowie and Cohen 1957; Voloshchuk and Montclare 2010).

Initially, the approach was used in the X-ray crystallography to solve phase problem necessary for structure determination – a heavy atom replacement (sulfur atom of methionine was substituted for selenium of selenomethionine) for subsequent multi-wavelength anomalous diffraction experiment (Hendrickson *et al.* 1990). The same principle was utilized in a completely different protein structure determination methodology – photo-initiated crosslinking – where the methionine analog containing photo-labile functional group is introduced (Suchanek *et al.* 2005).

The reassignment of methionine codon is widely used for protein engineering as the methionyl-tRNA synthetase possesses relatively relaxed substrate binding pocket – number

of methionine analogs have been successfully introduced into expressed proteins. The other aaRSs have much more strict specificity for the respective amino acid, but also sense codons *e.g.* for proline, phenylalanine, or tyrosine were successfully reassigned (Hendrickson *et al.* 2004). Moreover, introducing appropriate mutations modifying substrate binding site of an aaRS can augment host's capabilities to globally incorporate coveted amino acid analog (Liu *et al.* 2007).

In comparison to site-specific incorporation approaches, (1) the yield of protein is higher (generally 10-100 mg/l (Link and Tirrel 2005); but also several g/l were achieved via high-density fermentation), (2) almost total incorporation can be achieved depending on the procedure and utilized analog, and (3) the expression system requires less modification and is better suited for upscaling and large-scale protein production (Wang *et al.* 2012).

1.5. Mixed function oxygenase system

Non-specific mixed function oxygenase (MFO) system participates on biosynthesis of endogenous substances (*e.g.* cholesterol or hormones) as well as arranges activation of several prodrugs. Unfortunately, it can also inactivate drugs administered in their active form and biotransform chemical procarcinogens into active carcinogens (Guengerich 2005). Detailed structural knowledge of these enzymes contributes to development of more considerate and effective medications as well as inhibitors blocking undesired effects.

Proteins of MFO system are anchored to lipid membrane of endoplasmic reticulum or mitochondria in higher organisms (Bar-Nun *et al.* 1980; Omura and Morohashi 1995). Besides other roles, they are involved in the first phase of biotransformation, in which they facilitate the substrate detoxification (Guengerich 2005). The most commonly catalyzed reaction is substrate hydroxylation summarized in equation:

$$RH + O_2 + NADPH + H^+ \xrightarrow{MFO} ROH + H_2O + NADP^+ .$$

One atom of an oxygen molecule is reduced to water using 2 electrons and 2 protons while the other is introduced into the substrate molecule making it more hydrophilic (White and Coon 1980). This polar group constitutes a site for conjugation with highly hydrophilic molecule (*e.g.* sulfuric or glucuronic acid) in the second phase of biotransformation, which further increases the original molecule solubility in aqueous solvents to promote its excretion (Jančová and Šiller 2012).

Cytochrome P450 (P450) serves as a terminal oxidase responsible for substrate biotransformation. The electrons necessary for the reaction have to be provided to P450 stepwise by its redox partners. **NADPH: P450 reductase** arranges both transfers of an electron to P450 in the catalytic cycle. **Cytochrome b_5** (cyb5) is a facultative component of MFO system which can mediate transfer of the second electron to P450 instead of the reductase, but more importantly can modulate its catalytic properties. The interactions of both cytochromes have not been fully understood, but depend on P450 isoform, metabolized substrate, and experimental conditions (Schenkman and Jansson 2003; Zhang *et al.* 2005). The **phospholipid membrane** provides an environment in which MFO proteins can adopt correct mutual orientation appropriate for interaction. It is substantial component of eukaryotic monooxidase system and affects significantly its catalytic activity (Ingelman-Sundberg 1977).

1.5.1. Cytochrome P450

Cytochromes P450 were originally considered heme-containing liver pigments with extraordinary spectral properties – their reduced form with bound carbon monooxide shows characteristic absorption maximum at 450 nm (Omura and Sato 1964). The divergence from other hemeproteins with the absorption maxima of Soret band between 380 and 420 nm is caused by the thiolate sulfur coordinated to the central iron atom of protoporphyrin IX prosthetic group in the P450 active site (Murakami and Mason 1967; Dawson and Sono 1987).

The P450 superfamily consist of branched families and subfamilies; their designation consists of a number indicating family (more than 40 % sequence identity), a capital letter indicating subfamily (more than 55 % sequence identity), and another numeral indicating particular isoform (Nelson 2004). P450 2B subfamily members (*e.g.* rabbit isoform 2B4) have become prototypical model for studying plasticity of mammalian P450s. They exhibit low degree of catalytic conservation – the minor sequence divergence amongst individual subfamily members is associated with substantial difference in functionality (Zhao and Halpert 2007).

Mammalian microsomal P450s comprise approximately 500 amino acids, which build a large C-terminal catalytic domain and an N-terminal transmembrane anchor. The cytosol exposed domain typically consists of 12 major α-helices (A-L) and 3 β-sheets (1-3) forming shape of a triangular prism (Poulos *et al.* 1985). Six α-helices (C, D, and I-L)

together with two β-sheets (1-2) represent more conserved structural core where heme is localized (Poulos and Johnson 2005). The electron transfer to P450 prosthetic group is mediated by either NADPH: P450 reductase or cyb5 which binds to the patch of positively charged amino acids on the proximal surface. P450 is oriented differently towards each redox partner when the electron is being transferred as both binding sites are partially overlapping, but not identical (Bridges *et al.* 1998; Scott *et al.* 2004; Gao *et al.* 2006).

On the other hand, four α-helices (B-C and F-G) and C-terminus delineate more dynamic, and less conserved substrate binding cavity on the distal side of the protein (Poulos and Johnson 2005). It differs greatly in size, shape and plasticity, and dictates the specificity of individual P450 isoforms. While some highly specific isoforms take part in transformation of only few substrates of the same physico-chemical character, others are more universal and oxidize broad range of distinct molecules (Pylypenko and Schlichting 2004; Denisov *et al.* 2005). The active site is connected with the cytosol or lipid membrane by several channels, which allow substrates to access and products to leave the enzyme (Cojocaru *et al.* 2007).

The N-terminal part of eukaryotic P450s consists of approximately 20 highly hydrophobic amino acids followed by short polycationic segment, and anchoring them to lipid membrane (Williams *et al.* 2000). The role of this domain has not been fully explained yet, but its presence is essential for catalysis. It could be necessary for delivering hydrophobic substrates to the active site of P450, or could provide environment for MFO system components to assemble and interact in functional conformations (Causey *et al.* 1990). Additional peripheral membrane contacts are arranged by hydrophobic segments of α-helices A, and F-G (Von Wachenfeldt and Johnson 1995; Ozalp *et al.* 2006; Mast *et al.* 2009).

1.5.2. Cytochrome b_5

Cytochrome b_5 is small hemoprotein (~ 17 kDa) acting as an intermediate electron acceptor of either NADPH: P450 reductase or NADH: cyb5 reductase (Dürr *et al.* 2007). It can supply the second electron required in P450 catalytic cycle, and modulate activity and product profile of certain P450 isoforms (Vergéres and Waskell 1995; Gruenke *et al.* 1995). Additionally, cyb5 also provides reducing equivalents for lipid biosynthesis (Oshino and Omura 1973).

N-terminal heme-binding domain is exposed to cytosol and includes a surface patch composed of negatively charged amino acids and heme propionate carboxyles, which are complementary to positively charged residues of P450 at the interaction interface. However, the hydrogen bonds and van der Waals' interactions also contribute to complex formation (Tamburini *et al.* 1986).

Contrary to P450, two histidine residues coordinate the central iron atom of heme, which is responsible for different spectral properties (absorption maximum at 413 nm) (Strittmatter and Velick 1956). The C-terminal hydrophobic domain anchors cyb5 to the phospholipid membrane of endoplasmic reticulum, and its presence is essential for cyb5 ability to affect P450 catalysis (Vergéres and Waskell 1995). Both domains are connected by flexible linker that also influences the character of interaction between cyb5 and P450 (Clarke *et al.* 2004).

The effect of cyb5 on P450 depends on experimental conditions, presence or absence of a substrate, and on particular P450 isoform. For example, low concentration of cyb5 prevents formation of side products of the P450 2B4 catalytic cycle and stimulates its activity 10- to 100-fold (Zhang *et al.* 2007). On the other hand, cyb5 at high concentration competes with NADPH: P450 reductase for the overlapping P450 binding site and prevents donation of the first electron during the catalytic cycle, which inhibits the biotransformation (Schenkman and Jansson 2003). Cyb5 presence affects not only P450 activity but can also change the profile of P450 reaction products (Kotrbová *et al.* 2011).

Satisfactory amount of recombinantly expressed cyb5 can be obtained after an hour of production in routinely used bacteria strains (Kotrbová *et al.* 2009) and its expression is achievable also in the mineral medium, which conditions are suitable for incorporation of pMet into protein’s sequence. Thus, cyb5 can be easily turned into photo-reactive nanoprobe for exploration of interactions in lipid membrane as two methionines occur in hydrophobic domain (Koberova *et al.* 2013). In comparison, it takes 24 hours to produce P450 2B4 in a rich growth medium (Saribas *et al.* 2001), thus advanced expression system (*e.g.* suitable auxotrophic bacteria strain) and finely tuned growth medium would be required to obtain photo-reactive P450 2B4 nanoprobe.

2. AIM

1) Development of a novel photo-initiated cross-linking methodology for protein structure and protein-protein interactions determination

   - Optimization of amino acid photo-reactive analog (pMet) incorporation into cyb5 protein nanoprobe sequence during recombinant expression in *E. coli*

   - Photo-initiated covalent fixation of transient intermolecular interactions of P450 2B4 and photo-reactive cyb5

2) Application of chemical and photo-initiated cross-linking techniques and mass spectrometry to extend the present knowledge of protein structure

   - Localization of interactions between catalytic domains of cytochromes P450 2B4 and cyb5

   - Detection of presumed membrane contact of both cytochromes

3) Determination of mutual orientation(s) and stoichiometry of cyb5 and P450 2B4 in reconstituted system

3. RESULTS AND DISCUSSION

The catalytic characteristics of P450 2B4 are substantially modulated in the presence of its redox partner cyb5. The insight into the nature of their interactions is one of the fundamental steps towards detailed understanding of the whole multienzyme monooxygenase system. Both contacts of the cytosol exposed catalytic domains and cooperation between the hydrophobic anchors in the environment of lipid membrane were examined. The novel photo-initiated cross-linking methodology was established and optimized initially to probe the membrane topology of studied proteins, and subsequently to examine also the structure and behavior of their cytosolar domains. which are. The rather hydrophilic domains directly involved in the catalysis were also examined by well-established complementary chemical cross-linking technique. The interacting peptides were determined by high resolution MS, and the particular cross-linked sites were traced by tandem MS when possible. The presented findings were summarized in four research papers attached as Supplements 1-4.

3.1. Photo-initiated cross-linking technique development

The methionyl-tRNA synthetase makes an exception in stringent specificity of aaRSs, which safeguard correctness of genetic code translation. It activates not only Met but also the structurally closely resembling analogs, which are subsequently inserted into the sequence of newly synthetized protein (Kiick and Tirrel 2000). Suchanek *et al.* (Suchanek *et al.* 2005) cultivated mammalian cells in a medium supplemented with pMet (methionine analog containing photo-labile diazirine functional group). Subsequently, the photo-labile analogs integrated into the proteins were UV-activated *in vivo* and covalently linked protein assemblies were investigated by western blotting to determine individual interacting partners. We switched to less complex bacterial system – *E. coli* BL-21 (DE3) Gold – offering fast, cheap and undemanding protein expression. Besides, we introduced more sensitive analytical tool (MS) instead of western blot not only to determine the identity of individual interacting proteins but also to localize their contact sites. The modified methodology was described in a publication (Supplement 1):

> Koberova, M., **Jecmen, T.**, Sulc, M., Cerna, V., Hudecek, J., Stiborova, M., Hodek, P., 2013. Photo-cytochrome b5 – A New Tool to Study the Cytochrome P450 Electron-transport Chain. Int. J. Electrochem. Sci. 8, 125-134.

3.1.1. Optimization of photo-methionine incorporation into cytochrome b_5

Cyb5 was chosen as a suitable nanoprobe for studying structure of membrane domains of the mixed function oxygenase system proteins. It is a small membrane protein with rapid expression, relatively uncomplicated purification, and most importantly contains total of 3 methionines from which two are localized in the membrane domain and one is part of the polypeptide linker chain (**Figure 3.1A** on page 29).

Initially, the bacterial culture grown in LB medium to optical density (OD_{600}) ~1.0 was transferred to commercially available DMEM-LM limiting medium supplemented with either deuterium labeled Met (L-methionine-methyl-d3) or pMet for the protein production. Although the amount of cyb5 expressed in 4 hours observed on polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) was not markedly affected by the Met analog choice, its incorporation differed dramatically. Met was almost completely substituted by deuterated amino acid whereas only about 25% incorporation of pMet was observed according to MALDI-TOF MS.

The findings were not surprising for deuterated Met as it structurally differs minimally from the natural amino acid. The data for pMet were consistent with Suchanek *et al.* (Suchanek *et al.* 2005), who reported one in three Mets to be substituted by the photo-analog. The available residual Met (originating from methionine pool, degraded proteins, or *de novo* synthesis) can probably reduce or even fully suppress activation of unnatural analogs by MetRS as their K_D is in general 5 to 5500-fold greater than K_D of Met (Kiick and Tirrel 2000). Alas the constant has not been determined for pMet.

The protein production protocol was modified in several ways to moderate the competition of pMet for the activation by MetRS. First, the nutritionally rich LB medium used for cultivation was repeatedly and more rigorously rinsed out of the bacterial culture prior to its transfer to the limiting medium, where the protein was expressed. Next, the proteosynthesis was initialized after reaching OD_{600} ~0.6 to reduce the amount of old and dead cells, which could have been potential source of Met. Both alterations led to slight increase of pMet content in produced proteins. On the other hand, decreasing concentration of unnatural amino acid in limiting medium affected the degree of substitution minimally, thus its consumption could be at least 4 times lower than suggested by Suchanek *et al.* (Suchanek *et al.* 2005).

Increasing volume of *E. coli* culture in distinct vessels (10 ml in 50ml test tube, 30-40 ml in 50ml test tube or 250 ml in 1000ml erlenmeyer flask) cultivated at the

identical conditions led gradually to lower methionine substitution. The 40mL batches (6 at a time) were used in the most of the experiments as a compromise between the amount of produced nanoprobe and the pMet incorporation.

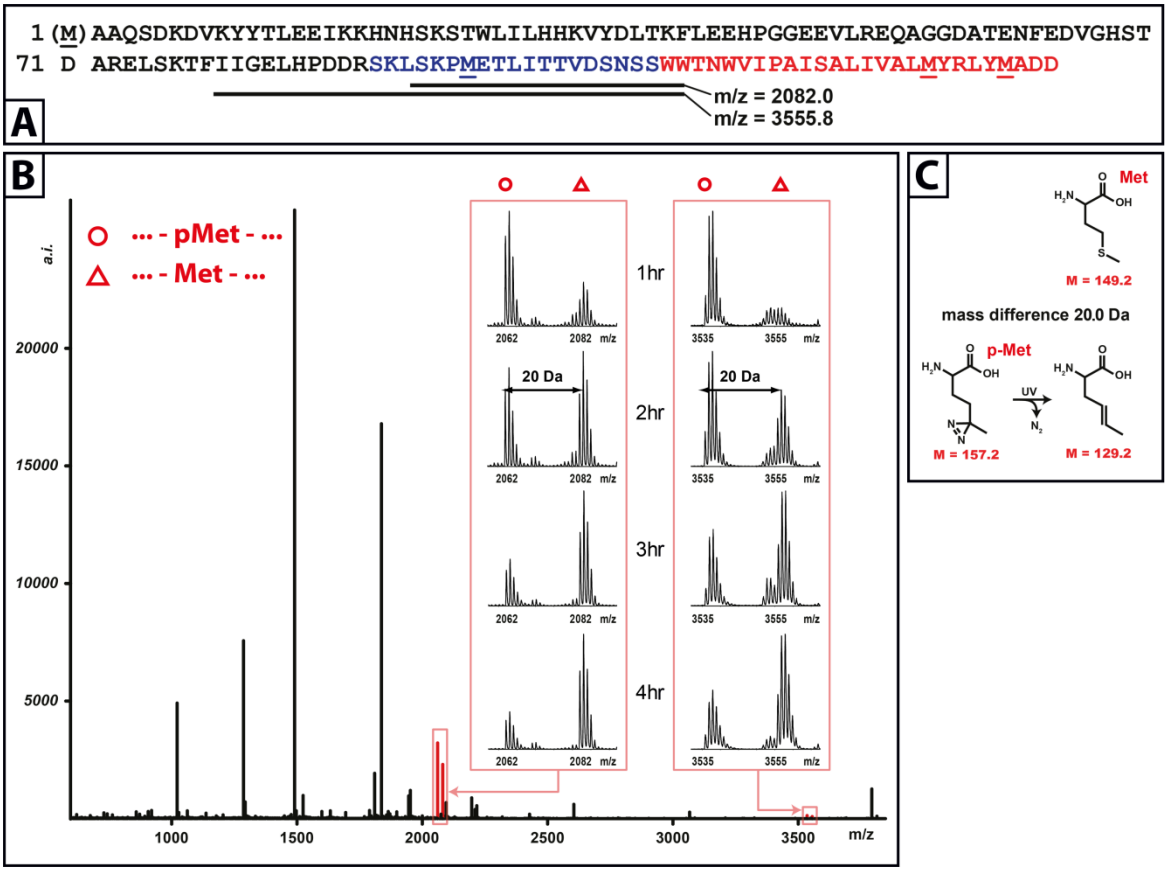

Figure 3.1: Incorporation of pMet into the cyb5 sequence. (A) The amino acid sequence of cyb5 WT with marked peptides selected for monitoring of pMet incorporation efficiency; black – cytosolar domain, blue – flexible linker, red – membrane anchor. (B) The time-dependence of pMet incorporation efficiency during the nanoprobe expression; MALDI-TOF mass spectra of photo-reactive cyb5 chymotryptic digest, in detail two peptide pairs with pMet/Met in the sequence, the ratio of their relative intensities expresses the pMet incorporation success rate. (C) MALDI laser activates pMet during the peptide ionization; the peptide pairs with either pMet photolytic product or Met typically differ by 20.0 Da in MALDI-TOF spectra.

The rate of pMet insertion into the nanoprobe exhibited decreasing tendencies from ~70 % after the first hour of expression to formerly observed ~30 % after 4 hours (**Figure 3.1B**) for different concentration of externally supplied pMet. The trend most probably expresses significant increase in the endogenous concentration of Met in the course of the experiment. *E. coli* cultivated in the rich medium have downregulated the enzymes of amino acid biosynthetic system and it takes bacteria about 1.5 hours to activate the biosynthetic pathway yielding Met (Zaslaver *et al.* 2004). This idle time corresponds with time span of the highest rate of pMet incorporation. It can also clarify observed poor incorporation of the photo-analog in the bacteria cultivated in the limiting instead of the

rich medium from the beginning because the amino acid biosynthetic pathways had already been activated at the time of cyb5 expression initiation.

Finally, six photo-reactive cyb5 mutants, each containing single methionine in the sequence, were prepared either to provide less complex cross-linking results compared to those obtained with the WT nanoprobe (the mutants with pMet at position 126 and 131 in the membrane anchor, and 96 in the flexible linker domain) or to extend the contemporary knowledge of the catalytic domain interactions (the mutants with pMet at the position 26, 41 and 46, which are not present in the WT protein). All prepared nanoprobes had over 30 % of the Met residues substituted with the photo-reactive analog, which was sufficient for photo-initiated cross-linking experiments.

3.2. Structural findings describing topology of cytochrome P450 2B4 and cytochrome b_5

The structure and mutual orientations of cyb5 and P450 2B4 were investigated by two complementing techniques based on zero-distance cross-linking. The interactions of cytosol exposed catalytic domains of both studied cytochromes were examined either by water soluble chemical agent EDC or utilizing photo-labile Met analog that was incorporated into protein nanoprobes (three cyb5 mutants with a Met site introduced into the catalytic domain). Only photo-initiated cross-linking can also address interactions in hydrophobic environment of lipid membrane. Photo-reactive cyb5 wild type and three corresponding mutants with single Met site (M96, M126 or M131) were used for searching contacts between transmembrane anchoring domains of both cytochromes. The cross-linked species were analyzed by high resolution and tandem MS and acquired structural data were used for homology modeling. Obtained results were described in one publication (Supplement 2) and one manuscript currently under revision (Supplement 3):

Sulc, M., **Jecmen, T.**, Snajdrova, R., Novak, P., Martinek, V., Hodek, P., Stiborova, M., Hudecek, J., 2012. Mapping of interaction between cytochrome P450 2B4 and cytochrome b5: the first evidence of two mutual orientations. Neuro. Endocrinol. Lett. 33(Suppl3), 41-47, and

Ječmen, T., Ptáčková, R., Černá, V., Dračínská, H., Hodek, P., Stiborová, M., Hudeček, J., Šulc, M., 2015. The Photo-Initiated Cross-linking Extends Mapping of Protein-Protein Interface to the Membrane-embedded Parts: Cytochromes P450 2B4 and b5. Methods, under revision.

3.2.1. Cross-linking experiments

Transient protein-protein interactions need to be transformed into covalent bonds to withstand experimental conditions of the analysis. EDC agent fixates electrostatic interactions between charged amino acids, whereas pMet conjugates frequently amino acids in less polar regions held together by the hydrophobic effect, where the Met sites naturally occurs. However, it can link to any residue in its close proximity due to radical character of the reaction. For the experiments, equimolar amounts of P450 2B4 and (photo-reactive) cyb5 were reconstituted with preformed lipid vesicles mimicking lipid membrane of endoplasmic reticulum, and cross-linked by one of the techniques.

First, the reconstituted system incubated with EDC chemical cross-linker for 10 hours yielded two heteromeric P450 2B4:cyb5 complexes visualized by Coomasie Brilliant Blue R-250 stain on SDS-PAGE (**Figure 3.2**). Their approximate molecular weights 70 kDa and 125 kDa roughly correspond with 1:1 and 2:1 protein ratios, respectively. The experiment was performed under varying concentration of sodium chloride (0 – 500 millimolar).

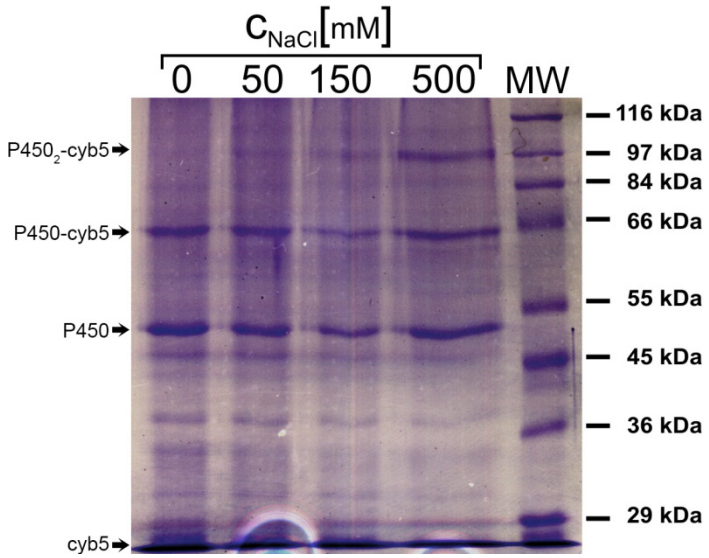

Figure 3.2. 1-dimensional electrophoretic separation of P450 2B4:cyb5 heterooligomers cross-linked by EDC agent. 8% SDS-PAGE, Coomassie Brilliant Blue R-250 staining; MW – Sigma wide-range molecular weight standards, cross-linking of cyb5 and P450 2B4 performed under different sodium salt concentrations (0, 50, 150 and 500 millimolar), the monomers and the chemically cross-linked complexes with different stoichiometry are labeled by arrows.

The formation of complex reminded undisturbed even under the highest tested salt concentration, which indicates also significant contribution of non-electrostatic interactions at the contact surface. The only near-physiological conditions inherent to the EDC chemistry (pyridine buffer, pH 6.0) was shown to decrease catalytic activity of the P450

2B4 specific O-depentylation reaction (Parmar *et al.* 1998). However, no change in the characteristic influence of cyb5 on P450 2B4 (the increased activity in the equimolar amount of cyb5 and the decreased activity in its excess) was observed, thus the mechanism of interaction was considered unaltered under the cross-linking compatible conditions.

Second, the reaction mixture containing reconstituted P450 2B4 and one of seven photo-reactive nanoprobes (cyb5 wild type and six mutants each containing single pMet) was photolyzed by a 100W mercury arc lamp with the emission maximum at 254 nm (the wavelengths below 300 nm responsible for protein damage were filtered out by Pyrex borosilicate glass). The proteins lacking photo-reactive amino acids were shown neither to cross-link spontaneously nor are degraded under the UV-irradiation conditions. Generally, half-lives of highly reactive carbene biradicals ranges from 0.5 millisecond to 0.5 nanosecond (Bayley 1983), thus photo-initiated cross-linking requisite much shorter time to proceed than does chemical cross-linking reaction, as it is completed in the range of minutes instead of hours. Three newly formed oligomeric covalent complexes with approximate molecular weights 70 kDa, 90 kDa and 125 kDa were detected on SDS-PAGE for all of the nanoprobes (**Figure 3.3**).

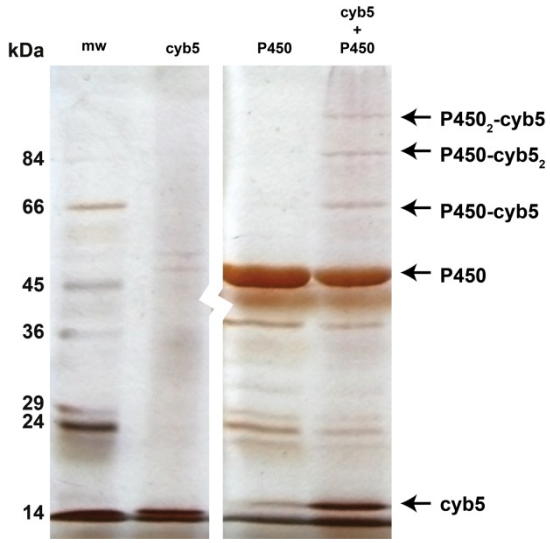

Figure 3.3. 1-dimensional electrophoretic separation of photo-cross-linked P450 2B4:cyb5 heterooligomers. 12% SDS-PAGE, silver staining; MW – Sigma wide-range molecular weight standards, reconstituted photo-reactive cyb5 and P450 2B4 photolyzed separately (two middle lanes) and together (right lane), the monomers and the photo-cross-linked complexes with different stoichiometry are labeled by arrows.

The most probable cyb5:P450 2B4 ratio in the individual complexes seemed to be 1:1, 2:1 and 1:2, respectively. However the stoichiometry confirmation turned up to be difficult, and is discussed in detail in **Chapter 3.3** on page 39.

In comparison with chemical cross-linking, the more sensitive silver staining was employed to visualize the cross-linked products due to their lower abundance. As an implication, the 4-fold excess of the nanoprobe was added to the reconstituted reaction mixture to obtain greater amounts of cross-linked species for MS analysis. Anomalous binding at higher cyb5 concentrations is not expected due to (i) the identical SDS-PAGE pattern for diverse protein ratios, and (ii) the general assumption that observed P450 2B4 catalytic activity inhibition caused by the cyb5 excess is due to its competition with NADPH: P450 reductase for the partially overlapping binding site on the P450 2B4 proximal surface (Zhang *et al.* 2007).

3.2.2. Mass spectrometric analysis

The combination of both above mentioned cross-linking techniques with MS is an especially valuable tool for examination of protein-protein interactions – it is realized at reasonable time-scales, with relatively small sample consumtion, and the most importantly observes proteins in their native states (Sinz 2006; Sinz 2014). The „bottom up“ approach was used for studying of P450 2B4:cyb5 complexes – they were separated on SDS-PAGE, proteolytically digested and the resulting peptide mixtures were analyzed by MS.

MALDI-TOF MS analysis verified the identity and purity of all proteins used for the experiments as well as confirmed the occurrence of both cytochromes in each complex by peptide mass fingerprinting – the *m/z* signals observed in the spectra were matched to the *m/z* values in the *in silico* generated library of theoretical peptides fitting to the specificity of employed protease. Further, MS was used for monitoring of pMet incorporation efficiency. The same peptides containing either Met or pMet differ by the mass of 8.034 Da, and the N_2 molecule is eliminated from the pMet containing peptide (the mass loss 28.006 Da) during its ionization by the MALDI laser emitting at 337 nm. Altogether, the *m/z* signals in the spectra differing by the mass of 19.972 Da indicate pMet/Met containing peptide pairs, and the ratio of their relative intensities express the success rate of the photo-analog incorporation into the nanoprobe (**Figure 3.1C** on page 29).

The reverse phase LC on-line coupled with the high-resolution ESI-FT-ICR mass spectrometer serves for the separation of unmodified, modified and cross-linked peptides, and their subsequent identification according to the accurately determined mass (error below 2 ppm). The obtained datasets of experimental *m/z* values were automatically

reduced to produce a list of monoisotopic masses that were searched against the database of theoretical monoisotopic masses of cross-linked products: the mass of cyb5 peptide plus the mass of P450 2B4 peptide minus either the mass of the water (the mass shift of 18.011 Da) eliminated in the process of a carboxyl and an amino group conjugation by EDC agent, or the mass of the N_2 molecule (the mass shift of 28.006 Da) eliminated from pMet as the consequence of photolysis. The assigned signals are summarized in **Table 3.1** for EDC and in **Table 3.2** on page 35 for pMet. Particular interacting amino acids are marked when the unambiguous determination could be deduced according to the known reaction mechanism (EDC cross-links a carboxylate group of aspartate, glutamate or C-terminus with an amine group of arginine, lysine or N-terminus).

Table 3.1. Intermolecular peptides of P450 2B4:cyb5 heterodimeric complex cross-linked by EDC, and identified by LC-ESI-FTICR MS.

Cross-linker	$[M+H]^+$ mass (Da)	Error (ppm)	cytochrome b_5 peptide	P450 2B4 peptide
EDC	2479.2371	0.4	8–10 K.<u>D</u>VK.Y	134–151 R.DFGMG<u>K</u>RSVEERIQEEAR.C
	2504.2917	1.6	19–24 K.<u>K</u>HNHSK.S	126-140 R.RFSLATMR<u>D</u>FGMGKR.S
	2657.4001	0.1	19–33 K.<u>K</u>HNHS<u>K</u>STWLILHHK.V	134-140 R.<u>D</u>FGMGKR.S
	1719.9058	1.0	19–24 K.<u>K</u>HNHSK.S	435-443 R.IC$^{(CAM)}$LG<u>E</u>GIAR.T
	3117.5918	1.2	20-33 K.HNHS<u>K</u>STWLILHHK.V	423-434 R.N<u>E</u>GFM$^{(ox)}$PFSLGKR.I

Legend: $M^{(ox)}$ marks an oxidized methionine, $C^{(CAM)}$ marks a cysteine modified with carbamidomethyl, the underlined amino acid labels the cross-linked residue, and the periods in the sequence delimit the identified peptide.

The identified intermolecular contacts of both cytochromes fall into three groups: (i) interactions of catalytic domains at the P450 2B4 proximal side, (ii) interactions of catalytic domains at the P450 2B4 distal side, and (iii) interactions between the hydrophobic anchors in the membrane.

The measurements of cyb5 dissociation constant for selected P450 2B4 mutants (Bridges *et al.* 1998), the chemical cross-linking of cyb5 with P450 2E1 isoform followed by MS (Gao *et al.* 2006), and the NMR spectroscopy experiments (Ahuja *et al.* 2013) showed a range of P450 residues involved in cyb5 binding. In the assumed orientation of the proteins the cyb5 heme prosthetic group is directed towards the heme prosthetic group of P450 2B4 localized close to its proximal surface. We successfully labeled additional

P450 2B4 amino acids participating in binding that fall into this region – peptides 86-98, 127-145, and 423-443 (**Figure 3.4A,B** on page 36, **Figure 3.5A,D** on page 37).

Table 3.2. Intermolecular photo-cross-linked peptides of P450 2B4:cyb5 oligomeric complexes identified by LC-ESI-FTICR MS.

Cross-linker	$[M+H]^+$ mass (Da)	Error (ppm)	cytochrome b_5 peptide	P450 2B4 peptide
photo-cyb5 WT	1350.6783	1.3	126–130 L.pMYRLY.M	1–5 -.MEFSL.L
	1338.7349	1.3	93–99 R.SKPpMETL.I	314–317 L.MLKY.P
	2287.2107	0.5	85–99 L.HPDDRSKLSKPpMETL.I	314–317 L.MLKY.P
photo-cyb5 mutM23	2038.0076	1.2	20-24 K.HNHpMK.S	86-98 R.EALVDQAEAFSGR.G
	2223.1307	1.8	20-24 K.HNHpMK.S	86-100 R.EALVDQAEAFSGRGK.I
	1420.7423	1.4	20-24 K.HNHpMK.S	140-145 K.RSVEER.I
	1548.836	0.4	19-24 K.KHNHpMK.S	140-145 K.RSVEER.I
	1837.9904	0.9	19-24 K.KHNHpMK.S	152-158 R.CLVEELR.K
	2172.1105	1.2	19-24 K.KHNHpMK.S	423-433 R.NEGFMPFSLGK.R
	1887.9174	0.2	20-24 K.HNHpMK.S	423-433 R.NEGFMPFSLGK.R
photo-cyb5 mutM41	2370.1721	1.5	40-52 K.FpMEEHPGGEEVLR.E	152-158 R.CLVEELR.K
photo-cyb5 mutM46	3125.6016	1.3	34-52 K.VYDLTKFLEEHPpMGEEVLR.E	127-133 R.FSLATMR.D
	2184.0974	1.9	40-52 K.FLEEHPpMGEEVLR.E	140-145 K.RSVEER.I
	2501.2527	0.2	40-52 K.FLEEHPpMGEEVLR.E	152-158 R.CLVEELR.K
	4914.317	1.3	40-73 K.FLEEHPpMGEEVLREQAG GDATENFEDVGHSTDAR.E	434-443 K.RICLGEGIAR.T

Legend: pM stands for the photo-labile analog of methionine, and the periods in the sequence delimit the identified peptide.

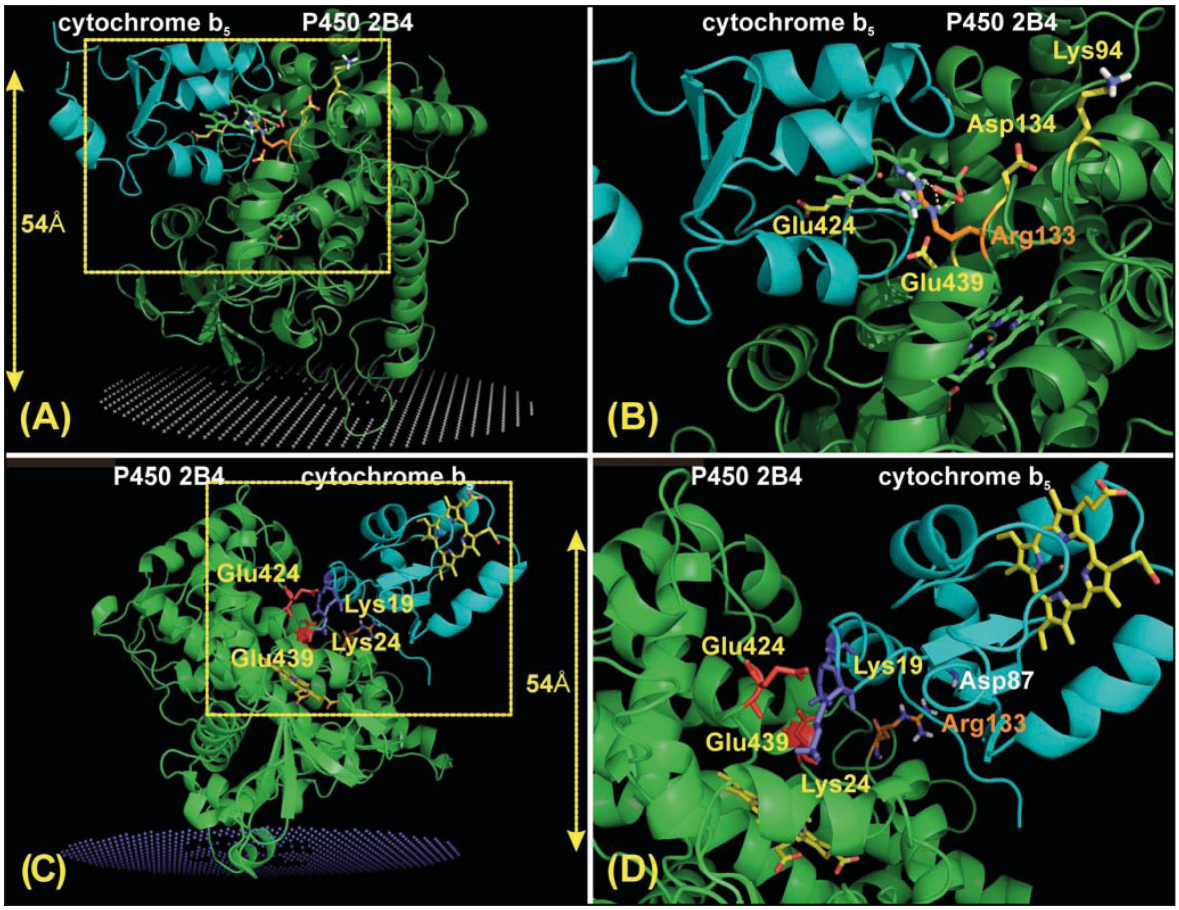

Figure 3.4. The homology model of P450 2B4:cyb5 interaction based on EDC cross-linking data. (A) and its detail (B) show the protein orientation suitable for electron transfer; the cyb5 heme prosthetic group is directed towards the heme prosthetic group of P450 2B4 localized close to its proximal surface. (C) and its detail (D) show cyb5 interacting with the proximal surface of P450 2B4, but with heme prosthetic group oriented out of this surface; the interaction may result only in allosteric modulation of P450 2B4 metabolic activity. Green – P450 2B4, cyan – cyb5, white lines – topology of lipid membrane.

However, molecular modeling using identified interactions Glu424 (P450 2B4)-Lys24 (cyb5) and Glu439 (P450 2B4)-Lys19 (cyb5) simultaneously as constrains proposes alternative protein topology – cyb5 still interacting with the proximal side of P450 2B4, but with the heme prosthetic group oriented out to the cytosol (**Figure 3.4C,D**). Not only the cross-linking data but also the calculated distance between the C-terminus of cyb5 and the phospholipid membrane presented in OPM database (Lomize *et al.* 2006) suits this novel orientation. Further, the peptide (152)CLVEELR(158) labeled by all three cyb5 mutants with Met site introduced into the catalytic domain as well as peptide (314)MLKY(317) labeled by pMet96 of cyb5 WT linker domain (**Figure 3.5B,C** on page 37) are localized on the P450 2B4 distal side, and suggest another cyb5 orientation never presented before. The yields of aforesaid cross-links were not sufficient to allow peptide sequencing by tandem MS, and only low structural data (on peptide instead of

residue level) were obtained. The protein topologies proposed on the basis of detected interactions do not allow the electron transfer between cytochromes, however can represent the protein orientations, in which P450 2B4 is allosterically modulated by cyb5.

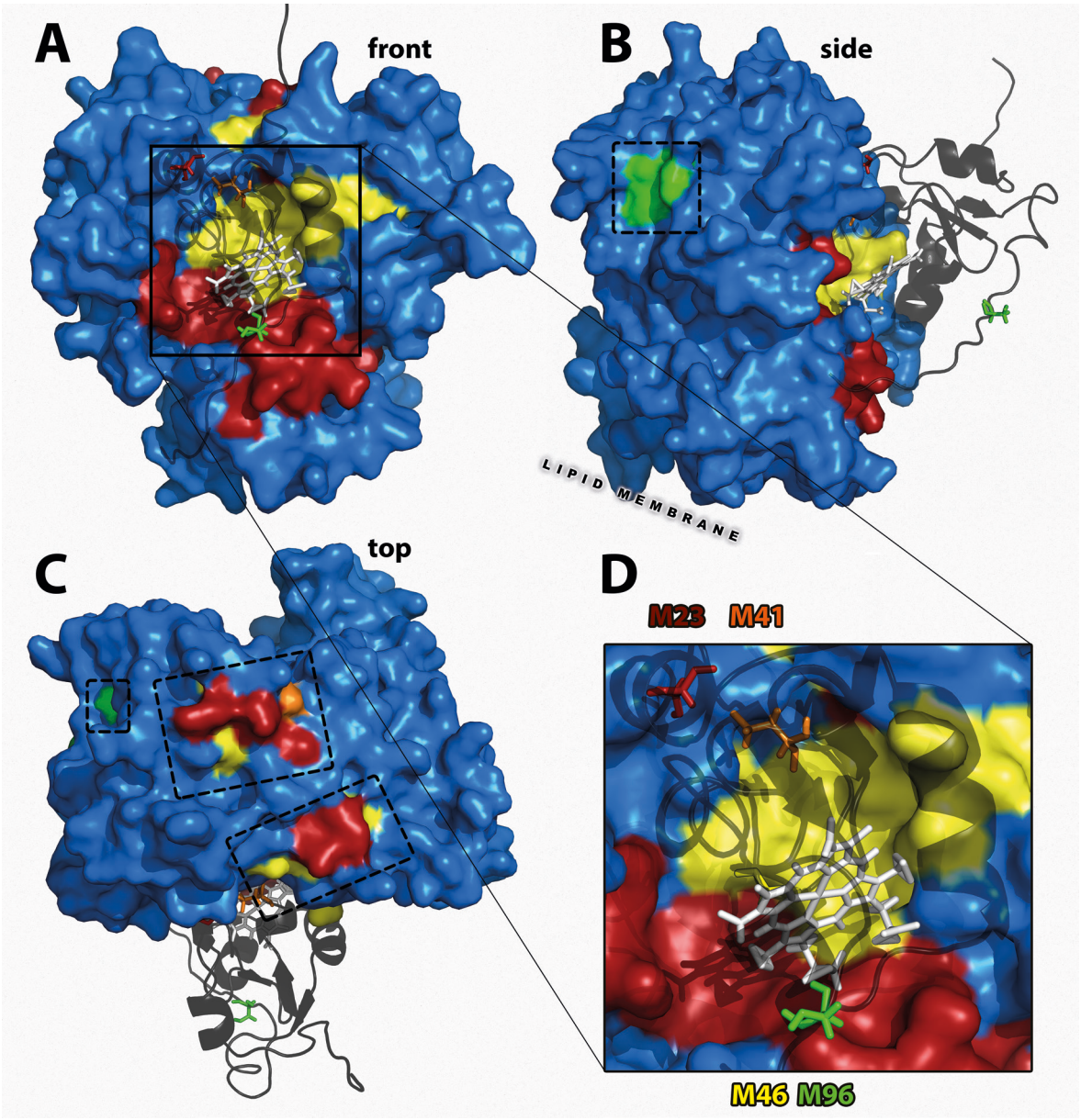

Figure 3.5. Vizualization of P450 2B4 peptides photo-labeled by photo-reactive cyb5. (A) Front, (B) side and (C) top view of P450 2B4 (blue) with peptides photo-labeled by pMet23 (red), pMet41 (orange), pMet46 (yellow) and pMet96 (green) of cyb5. Cyt5 (black, semitransparent) oriented suitably for electron transfer, (D) with the heme prosthetic group (white) directed toward the proximal surface of P450 2B4. Other cyb5 orientations responsible for photo-labeling of peptides marked with dashed line not shown.

Both cytochromes are tightly anchored to the phospholipid bilayer each by a single transmembrane helix (Scott *et al.* 2001; Banci *et al.* 2000). The solid state NMR demonstrated that the interaction of both cytochromes neither alters helical structure of their membrane domains nor changes their geometry (Yamamoto *et al.* 2013). However,

more detailed structural data are contemporarily missing due to the lack of convenient methods addressing the environment of lipid membrane. Therefore the discovered contact of P450 2B4 N-terminal peptide (1)MEFSL(5) with pMet126 localized at the cyb5 C-terminus is of a great importance: (1) it directly confirms MFO system protein-protein interaction in membrane environment, and (2) demonstrates the applicability of the photo-initiated cross-linking technique for probing topology of membrane proteins.

The monomers of photo-activated cyb5 mutants were also analyzed for intramolecular contacts. Only pMet introduced into the flexible loops of catalytic domain (pMet23, pMet41, and pMet46) provided positive cross-linking results (**Table 3.3**). The same interacting peptides were covalently bonded in all three mutants; however different amino acids were interlinked. The deduced low resolution structural data are in the agreement with the 3-dimensional structure of cyb5. No intramolecular contacts either for cyb5 or P450 2B4 were captured by EDC agent and no covalent homodimeric products were observed on the SDS-PAGE for either chemical or photo-initiated cross-linking.

Table 3.3. Intramolecular photo-cross-linked peptides of cyb5 monomer identified by LC-ESI-FTICR MS.

Cross-linker	$[M+H]^+$ mass (Da)	Error (ppm)	cytochrome b_5 peptide (1)	cytochrome b_5 peptide (2)
photo-cyb5 mutM23	2157.0876	1.9	20-24 K.HNHpMK.S	40-52 K.FLEEHPGGEEVLR.E
photo-cyb5 mutM41	2131.0362	2.1	20-24 K.HNHSK.S	40-52 K.FpMEEHPGGEEVLR.E
photo-cyb5 mutM46	2187.0941	0.8	20-24 K.HNHSK.S	40-52 K.FLEEHPpMGEEVLR.E
	2315.1891	1.3	19-24 K.KHNHSK.S	40-52 K.FLEEHPpMGEEVLR.E

Legend: pM stands for the photo-labile analog of methionine, and the periods in the sequence delimit the identified peptide.

3.2.3. Comparison of applied cross-linking techniques

The partially complementing chemical and photo-initiated cross-linking techniques were employed to probe interactions between two membrane bound cytochromes. Except for providing unique structural information, either also manifested its main advantages within this study.

Water-soluble EDC agent successfully fixated electrostatic interactions between the cytosol exposed catalytic domains of both proteins, the interacting peptides were identified

by MS, and particular cross-linked amino acids were determined – partly on the basis of the acquired tandem MS data, partly according the known EDC reaction mechanism.

In contrast, photo-initiated cross-linking demonstrated one of its major assets, and captured interactions both in cytosol and in the lipid membrane. For this purpose several cyb5 mutants were prepared by SDM, each containing single methionine site at the specific locations. The possibility to introduce the photo-reactive amino acid anywhere within the protein makes the technique suitable for screening of contact surfaces.

Compared to chemical cross-linking, in which the conditions were dictated by the optimum of EDC agent (pH 6.0, pyridine buffer), photo-initiated cross-linking was performed under the native conditions, as the radical reaction have no specific requirements to proceed. The nonselectivity of highly reactive biradicals arisen from photolysis means a valuable feature and a difficulty at the same time – pMet can link to any residue in its proximity, but subsequent identification of interacting amino acid pair depends entirely on the experimental data. As the yield of photo-cross-links is markedly lower than of their chemical counterparts, the fragment spectra were not acquired for any of photo-cross-linked species, and thus only the low resolution structural data (on a peptide level) were obtained by this technique.

3.3. Protein stoichiometry in cytochrome P450 2B4:cytochrome b_5 covalent complexes

The chemical agents react relatively slowly, which allow the rearrangement of protein molecules in the course of the cross-linking experiment. The fixation of individual transient interactions takes place at the different time points, and multiple protein orientations are captured by this approach. In comparison, the highly reactive biradicals formed during the photolysis connect proteins all at once and capture mostly simultaneous interactions of the studied system. On that account, the molar ratios of the proteins in photo-cross-linked covalent complexes as a snapshot of the studied system contribute substantially to the understanding of its organization and functioning.

The stoichiometry of the cytochromes in certain assemblies formed during the photolysis could not be conclusively determined according either 1-dimensional SDS-PAGE or MS measurements, and other methodological approaches (2-dimensional SDS-PAGE, Edman degradation and total amino acid analysis) were employed for this

purpose. The results solving protein ratios in the individual complexes and the existence of at least 2 distinct protein orientations are summarized in a publication (Supplement 4):

> **Ječmen, T.**, Ptáčková, R., Kavan, D., Černá, V., Hodek, P., Stiborová, M., Hudeček, J., Šulc, M., 2014. Quantification of interactions between cytochrome P450 2B4 and cytochrome b5 in a functional membrane complex. Neuro. Endocrinol. Lett. 35(Suppl2), 114-122.

3.3.1. Electromigration techniques

1-dimensional SDS-PAGE of the photo-activated reaction mixture (described above in **Chapter 3.2.1** on page 31) revealed both monomers – photo-reactive cyb5 nanoprobe and P450 2B4 – as well as 3 novel covalent protein complexes with approximate molecular weights 70 kDa, 90 kDa, and 125 kDa (**Figure 3.3** on page 32). The first two were easily interpreted as P450 2B4:cyb5 in molar ratios 1:1 (molecular weight 71 kDa) and 1:2 (molecular weight 86 kDa), respectively. The stoichiometry of the last one remained undecided – either protein ratio 1:3 (molecular weight 102 kDa), 1:4 (molecular weight 119 kDa) or 2:1 (molecular weight 127 kDa) were evaluated. The regular migration of proteins on SDS-PAGE can be altered for cross-linked species, thus the molecular weight determination based entirely on the mobility of the molecular standards is intricate, especially for large assemblies.

Both cytochromes differ in pI dramatically (value 5.1 calculated for cyb5 versus value 9.0 calculated for P450 2B4), and so do their covalent complexes with different stoichiometry (values 5.9 and 5.8 calculated for the respective 1:3 and 1:4 protein assemblies compared to value 8.2 calculated for the 2:1 protein assembly) (Bjellqvist *et al.* 1993). Therefore, 2-dimensional SDS-PAGE (**Figure 3.6** on page 41), which combines separation of analyzed species according to both electrophoretic mobility and isoelectric point (pI), was employed to distinguish between individual oligomeric complexes.

The approximate pI 6.6 assessed to the complex with expected stoichiometry 1:1 is in a good agreement with the calculated value 6.7. The protein stoichiometry 1:2 with calculated pI value 6.2 was represented by two distinct spots with slightly different electrophoretic mobility and assessed pI 5.9 and 6.1. It is likely that it reflects the aberrant behavior of dissimilarly cross-linked complexes with the identical protein ratio on SDS-PAGE. After proteolytic digestion, the peptides of both studied proteins were identified in each of the above mentioned spots by MS analysis.

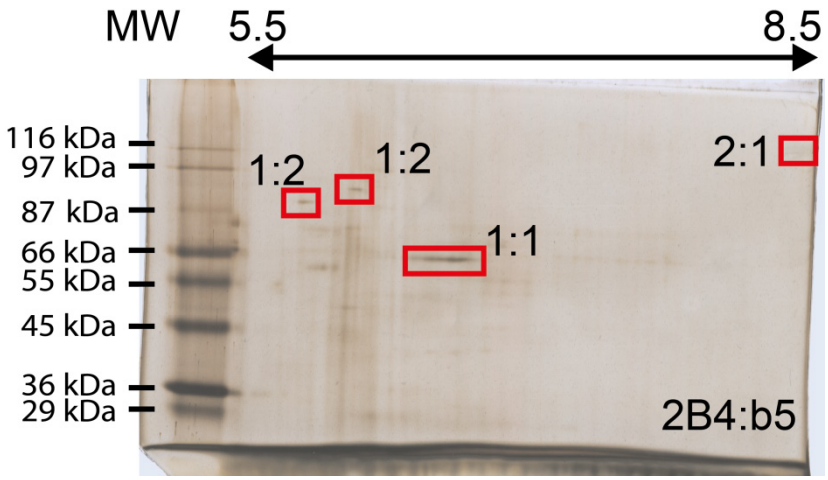

Figure 3.6. 2-dimensional electrophoretic separation of photo-cross-linked P450 2B4:cyb5 heterooligomers. 10% SDS-PAGE, pI range 5.5 – 8.5, silver staining; MW – Sigma wide-range molecular weight standards, the complexes with different stoichiometry are labeled, both monomers are missing (out of used pI range).

A protein spot with approximate molecular weight 125 kDa and pI 8.0 matching the calculated values for 2:1 protein assembly was also observed on silver stained gel. Unfortunately, its intensity was low and the spot did not provide adequate amount of material for verification of its composition. To sum up, neither 1-dimensional nor 2-dimensional electrophoresis convincingly tackled the stoichiometry of the largest protein assembly.

3.3.2. Amino acid composition determination

Two methods addressing amino acid composition, which is characteristic for each of the examined covalent complexes, were employed in an attempt to quantify the cytochromes. The separation of complexes on 1-dimensional SDS-PAGE and their subsequent western blotting to polyvinylidene fluoride (PVDF) membrane was necessary prior either technique.

Edman degradation cleaves an amino acid from each polypeptide chain of a particular heteromeric complex per sequencing cycle. Theoretically, several consecutive N-terminal residues can be identified and relatively quantified by this approach.

First, the excised PVDF membrane pieces of both monomers were analyzed together to determine the detection limit of this technique. Unfortunately, already the data acquired for these major components of the reaction mixture revealed only the qualitative information (the N-terminal amino acid sequences of both proteins), but were insufficient for quantitation. Thus, the much less abundant photo-reactive cross-linked products were also below the quantitative capabilities of this technique, and were not analyzed.

Total amino acid analysis hydrolyzes polypeptides and releases free amino acids, which are subsequently derivatized and inspected all at once. Similarly as in case of N-terminal sequencing, the modified amino acids are identified according to the characteristic retention times in LC and their quantities correspond to the areas under chromatographic curve (AUC). The direct hydrolysis of excised SDS-PAGE bands containing the complexes of interest introduced into the resulting samples high amount of ammonia, which originated from polyacrylamide. This interfering signal not only overlapped signals of several analyzed amino acids but also considerably decreased the sensitivity of the method (**Figure 3.7A**). This interference was eliminated by electroblotting of the complexes to PVDF membrane prior hydrolysis (**Figure 3.7B**).

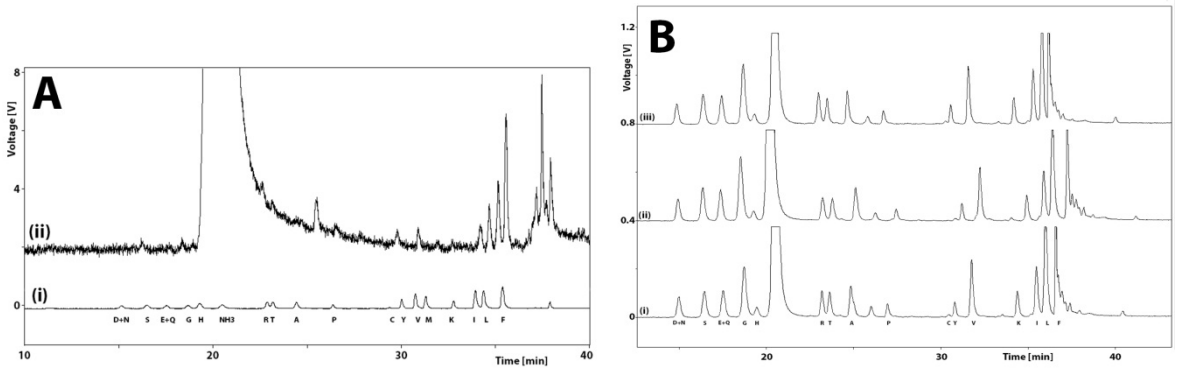

Figure 3.7. High performance LC chromatograms displaying the quantities of modified amino acids. (A) The separation of (i) equimolar standard mixture of amino acids, and (ii) hydrolyzed P450 2B4:cyb5 dimer excised from polyacrylamide gel. (B) The separation of P450 2B4:cyb5 heteromers with molecular weights (i) ~70 kDa, (ii) ~90 kDa, and (iii) ~125 kDa electroblotted to PVDF membrane after 1-dimensional SDS-PAGE. The amino acids are labeled by one letter abbreviations, NH3 stands for ammonia.

The AUC values characterizing particular amino acids were determined for all three oligomeric protein complexes as well as for both monomers. The values were normalized according to the calculated number of respective amino acids in a specie of expected stoichiometry (four possible compositions of the largest complex were taken into consideration) and related to the normalized AUC values of one of the monomers with known amino acid composition. This approach validated the proposed stoichiometry of the P450 2B4:cyb5 complexes with molecular weight ~70 kDa (1:1) and ~90 kDa (1:2), as well as resolved the protein ratio of the assembly with molecular weight ~125 kDa (2:1). Other proposed compositions (1:3, 1:4 and 1:5) were excluded for this complex as they significantly differed in one or more parameters characterizing the specie.

4. CONCLUSIONS

1) An uncanonical amino acid bearing photo-labile functional group (pMet) was successfully incorporated into the protein sequence of cyb5 and the whole procedure was optimized.

2) P450 2B4 was covalently interlinked with photo-reactive cyb5 nanoprobe (with methionine sites in the membrane and linker domains) by photo-initiated cross-linking methodology. The resulting heteromeric complexes were analyzed by high resolution MS and the membrane interactions were captured for the first time by this approach.

3) New methionine sites were introduced into the catalytic domain of cyb5 by SDM, and photo-reactive cyb5 mutants were expressed. Catalytic domains of both studied cytochromes were photo-cross-linked. The residues on P450 2B4 proximal surface known to interact with cyb5 were supplemented with additional interacting peptides and yet unexplored contact regions were identified.

4) Chemical cross-linking employing zero-length agent EDC, and followed by high resolution and tandem MS further extended the range of amino acids known to participate in binding of catalytic domains of P450 2B4 and cyb5. The acquired data served as a basis for *in silico* modeling of the interaction.

5) The protein stoichiometries of individual photo-cross-linked heteromeric complexes (P450 2B4:cyb5 – 1:1, 1:2, and 2:1) were deduced making use of electrophoretic techniques (1- and 2-dimensional SDS-PAGE) and a method for determination of total amino acid composition.

6) At least two distinct mutual orientations of studied proteins were proposed taking all presented findings (the results of both chemical and photo-initiated cross-linking experiments analyzed by high resolution MS, and the determination of protein ratio in individual heterooligomeric complexes) into consideration.

5. LIST OF PUBLICATIONS

[1] Sulc, M., **Jecmen, T.**, Snajdrova, R., Novak, P., Martinek, V., Hodek, P., Stiborova, M., Hudecek, J., 2012. Mapping of interaction between cytochrome P450 2B4 and cytochrome b5: the first evidence of two mutual orientations. Neuro. Endocrinol. Lett. 33(Suppl3), 41-47. IF 0.932

[2] Koberova, M., **Jecmen, T.**, Sulc, M., Cerna, V., Hudecek, J., Stiborova, M., Hodek, P., 2013. Photo-cytochrome b5 – A New Tool to Study the Cytochrome P450 Electron-transport Chain. Int. J. Electrochem. Sci. 8, 125-134. IF 3.729

[3] **Ječmen, T.**, Ptáčková, R., Kavan, D., Černá, V., Hodek, P., Stiborová, M., Hudeček, J., Šulc, M., 2014. Quantification of interactions between cytochrome P450 2B4 and cytochrome b5 in a functional membrane complex. Neuro. Endocrinol. Lett. 35(Suppl2), 114-122. IF 0.935

[4] **Ječmen, T.**, Ptáčková, R., Černá, V., Dračínská, H., Hodek, P., Stiborová, M., Hudeček, J., Šulc, M., 2015. The Photo-Initiated Cross-linking Extends Mapping of Protein-Protein Interface to the Membrane-embedded Parts: Cytochromes P450 2B4 and b5. Methods, under revision. IF 3.221

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Supplement 1

Koberova M, **Jecmen T**, Sulc M, Cerna V, Hudecek J, Stiborova M, Hodek P.

Photo-cytochrome b5 – A New Tool to Study the Cytochrome P450 Electron-transport Chain

International Journal of Electrochemical Science 8, 125-134 (2013)

The extent of participation: cooperation on recombinant expression and purification of photo-reactive cyb5, optimization of protein production protocol to increase pMet incorporation rate, realization of photo-initiated cross-linking experiment, MALDI-TOF analysis

Supplement 2

Sulc M, **Jecmen T**, Snajdrova R, Novak P, Martinek V, Hodek P, Stiborova M, Hudecek J.

Mapping of interaction between cytochrome P450 2B4 and cytochrome b5: the first evidence of two mutual orientations

Neuroendocrinology Letters 33 (Suppl3), 41-47 (2012)

The extent of participation: cooperation on protein purification, monitoring the influence of cyb5 on P450 activity, realization of chemical cross-linking experiment, assisting in high resolution MS data interpretation

Supplement 3

Ječmen T, Ptáčková R, Černá V, Dračínská H, Hodek P, Stiborová M, Hudeček J, Šulc M.

The Photo-Initiated Cross-linking Extends Mapping of Protein-Protein Interface to the Membrane-embedded Parts: Cytochromes P450 2B4 and b5

Methods, submitted

The extent of participation: recombinant expression and purification of photo-reactive cyb5 mutants, realization of photo-initiated cross-linking experiment, MALDI-TOF analysis, high resolution MS data interpretation, help with scientific paper finalization

Supplement 4

Ječmen T, Ptáčková R, Kavan D, Černá V, Hodek P, Stiborová M, Hudeček J, Šulc M.

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

Neuroendocrinology Letters 35 (Suppl2), 114-122 (2014)

The extent of participation: recombinant expression and purification of photo-reactive cyb5 mutants, realization of photo-initiated cross-linking experiment, Edman sequencing, cooperation on total amino acid analysis, MALDI-TOF analysis, high resolution MS data interpretation, help with scientific paper finalization