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.