Exploring the Interactome of Cytochrome P450 2E1 in Human Liver Microsomes with Chemical Cross-Linking Mass Spectrometry

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ABSTRACT:

This study aimed on exploration of the system-wide effects of the alcohol-induced increase in the content of cytochrome P450 2E1 (CYP2E1) in the human liver on drug metabolism. Using membrane incorporation of purified CYP2E1 modified with photoreactive crosslinkers benzophenone-4-maleimide (BPM) and 4-(N-succinimidylcarboxy)benzophenone (BPS), we explored the array of its protein-protein interactions (proteome) in human liver microsomes (HLM) with chemical cross-linking mass spectrometry (CXMS). Exposure of bait-incorporated HLM samples to light was followed by isolation of the His-tagged bait protein and its cross-linked aggregates on Ni-NTA agarose. Analyzing the individual bands of SDS-PAGE slabs of thereby isolated protein with the toolset of untargeted proteomics, we detected the cross-linked dimeric and trimeric complexes of CYP2E1 with other drug-metabolizing enzymes. Among the most extensively cross-linked partners of CYP2E1 are cytochromes P450 2A6, 3A4, 2C9, and 4A11. We also detected the conjugates of CYP2E1 with UDP-glucuronosyltransferases (UGTs) 1A6, 1A9, 2B4, 2B15, and 2B17. These results demonstrate the exploratory power of the proposed CXMS strategy and corroborate the concept of tight functional integration in the human drug-metabolizing ensemble through protein-protein interactions of the constituting enzymes. Of particular interest is the observation of efficient cross-linking of CYP2E1 with CYP4A11. This enzyme plays a central role in the synthesis of vasoactive eicosanoids and its interactions with alcohol-inducible CYP2E1 may shed light on the mechanisms of alcohol-induced hypertension.

1. Introduction

The major role in metabolism of drugs and other xenobiotics in human body is played by the cytochrome P450 ensemble, which is responsible for the metabolism of over 75% of all marketed drugs and new drug candidates [1-2]. A functional versatility of the cytochrome P450 ensemble is achieved through the presence of over a dozen of cytochrome P450 species differing in their substrate specificity. Although the premise that the properties of this ensemble represent a simple aggregate of the properties of the constituting P450 enzymes continues to be the cornerstone of a rational analysis of the routes of drug metabolism, its validity became essentially compromised [3-7]. An important complexity in the relationship between the composition of the pool of cytochromes P450 in human liver and the system-wide properties of the drug-metabolizing system is brought in by the consequences of protein-protein interactions of its components. These interactions include the formation of heteromeric complexes of multiple P450 species and the interactions of cytochromes P450 with other drug-metabolizing enzymes and potential regulatory proteins [8-12].

Of particular practical significance is the role played by protein-protein interactions of cytochromes P450 in alteration of human drug metabolism by chronic alcohol exposure. The multifold increase in the content of cytochrome P450 2E1 (CYP2E1) in liver and other tissues observed in both alcoholics and moderate alcohol consumers represents one of the most important effects of alcohol on protein expression [13-14]. Importance of this enzyme for the mechanisms of hepatotoxicity is well recognized [13]. Conversely, the involvement of CYP2E1 in the instances of severe pharmacokinetic and pharmacodynamic interactions of alcohol with drugs is commonly considered insignificant due to a minor role played by CYP2E1 in drug metabolism [15-16].

However, the impacts of alcohol-induced increase in CYP2E1 content in human liver on drug metabolism and other functions of the cytochrome P450 ensemble appear to be underestimated. The effects of interactions of CYP2E1 with other P450 enzymes provide the most likely explanation for the alcohol-induced increase in the metabolism of diazepam and doxycyclin[17-19], the substrates of CYP3A, or phenytoin, tolbutamide, and warfarin[20-21] metabolized primarily by CYP2C9. A conclusive evidence of a direct cause-to-effect relationship between alcohol-dependent induction of CYP2E1 and the effects of this kind is provided by our studies of the impact of CYP2E1 on the activity of CYP3A4, CYP1A2 and CYP2C19 in HLM[22-23].

The aim of the present study is exploring the network of protein-protein interactions (the interactome) of CYP2E1 in the ER of liver cells, which knowledge is ultimately needed for in-depth understanding of the effects of alcohol on drug metabolism and other functions of the cytochrome P450 ensemble. To this aim we employed chemical crosslinking mass-spectrometry (CXMS) as a powerful tool for studying the protein interactome. The strategy of our experiments is based on membrane incorporation of purified CYP2E1 modified with photoreactive crosslinkers benzophenone-4-maleimide (BPM) and 4-(N-succinimidylcarboxy)benzophenone (BPS). Exposure of bait-incorporated HLM samples to light was followed by isolation of the His-tagged bait protein and its cross-linked aggregates on Ni-NTA agarose. Analyzing the individual bands of SDS-PAGE slabs of thereby isolated protein with the toolset of untargeted proteomics allowed us to detect its crosslinked complexes with other drug-metabolizing enzymes (DMEs). Among the most extensively cross-linked partners of CYP2E1 are the cytochromes P450 2A6, 3A4, 2C9, and 4A11, as well as UDP-glucuronosyltransferases (UGTs) 1A6, 1A9, 2B4, 2B15, and 2B17. These results demonstrate the high exploratory power of the proposed CXMS strategy and corroborate the concept of tight functional integration in the human drug-metabolizing ensemble through protein-protein interactions of the constituting enzymes.

2. Materials and Methods

2.1 Chemicals

Benzophenone-4-maleimide (BPM) and Igepal CO-630 were the products of Sigma Aldrich Inc (St. Louis, MO). 4-(N-succinimidylcarboxy)benzophenone (BPS) was obtained from Chem-Impex Intl. Inc. (Wood Dale, IL), respectively. Pierce™ Trypsin Protease, MS Grade was purchased from Thermo Fisher Scientific. All other reagents were of ACS grade and used without additional purification.

2.2. Protein expression and purification

C-terminally His-tagged and N-terminally truncated Δ3-20 CYP2E1 [24] was expressed in E. coli TOPP3 cells and purified as described earlier[23].

2.3. Pooled human liver microsomes

In this study we used two different lots of Human Liver Microsomes (HLM) obtained from 50 donors (mixed gender), namely the lots LFJ and LBA designated hereafter as HLM(LBA) and HLM(LFJ). The relative abundances of 11 major cytochrome P450 species in both lots were characterized in our earlier study [25]. The supplier-provided characterization of both lots may be found in the Supplementary Materials to the above publication.

2.4. Characterization of the content of protein and cytochromes P450 in HLM

Determinations of protein concentrations in microsomal suspensions were performed with the bicinchoninic acid procedure [26]. The total concentration of cytochromes P450 in HLM was determined with a variant of the "oxidized CO versus reduced CO difference spectrum" method described earlier [23].

2.5. Modification of CYP2E1 with BPM and BPS.

The reaction with BPM was performed in 0.5 M K-phosphate buffer, pH 7.4, containing 20% glycerol. The modification with BPS was carried out in 0.125 M K-phosphate buffer, pH 8.2, containing 10% glycerol. Buffer replacement was carried out by a passage through a spin-out column of Bio-Gel P6 (Bio-Rad, Hercules, CA, USA) equilibrated with the buffer of choice and followed with a dilution with the same buffer to the final protein concentration of 10 μM. Resulting protein solution was placed into a conic glass vial and saturated with argon through gentle bubbling of the gas. In the case of reaction with BPS, at this stage we supplemented the incubation mixture with 0.2% Igepal CO-630 added as 10% solution in the same buffer. The modifying reagent (BPM or BPS) was added to a desired molar ratio to the protein (see Results) as 10 mM solution in dimethylformamide. The incubation vial was flushed with argon, tightly closed, and set for overnight incubation in dark at 4 °C with continuous siring. In the case of BPM, the reaction was stopped by adding reduced glutathione to the concentration of 1 mM. The detergent present in the incubation mixture with BPS was removed with the use of a DetergentOUT™ spin column (G-Biosciences, St Louis, MO). The protein was concentrated to 25-30 μM with the use of a Centrisart I MWCO 100 kDa concentrator (Sartorius AG) and passed through a spin-out column of Bio-Gel P-6, equilibrated with the protein storage buffer (0.1 M Hepes-HCl, 10% glycerol, 150 mM KCl).

2.6. Incorporation of benzophenone-modified CYP2E1 into HLM

Incorporation of benzophenone-modified CYP2E1 into HLM was performed following the procedure described previously [23,25,27]. Undiluted suspensions of HLM (20-25 mg/ml protein, 10-13 mM phospholipid) in 125 mM K-Phosphate buffer containing 0.25 M Sucrose were incubated with benzophenone-modified protein or intact purified CYP2E1 (in control experiments) for 16 - 20 hours in dark at 4°C under an argon atmosphere at continuous stirring. The incubation time was adjusted based on the experiments with monitoring the process of

incorporation with FRET-based techniques [23,25,27]. The protein being incorporated was added in the amount of one molar equivalent to the endogenous cytochrome P450 present in HLM. Following the incubation, the suspension was diluted 4-8 times with 125 mM K-Phosphate buffer, pH 7.4 containing 0.25 M sucrose, and centrifuged at 150,000 g for 90 min at 4 °C. The pellet was resuspended in the same buffer to the protein concentration of 15-20 mg/ml.

2.7. Photo-crosslinking of the bait protein and its subsequent isolation from HLM

The suspension of HLM with incorporated benzophenone-modified CYP2E1 was diluted to the protein concentration of 5 mg/ml by argon-saturated 0.125 M K-phosphate buffer, pH 7.4, containing 20% glycerol, 0.5 mM EDTA, and 200 mM Sucrose, and placed into 1x1 cm optical quartz cell. The cell was flushed with argon gas, tightly closed and exposed to a broadband UV-Vis light using a 6427 Xe flash lamp light source (Oriel Instruments, Stratford, CT) operating at 75 Hz flash rate and maximal power. After 2 hours of light exposure, the suspension was centrifuged at 105000 g for 90 mins. The supernatant was discarded and the pellet was re-suspended in 1 mL of 0.125 M K-phosphate buffer, pH 7.4, containing 20% glycerol and 0.5% Igepal CO-630. The mixture was incubated for 2 hours at 4°C at continuous stirring and centrifuged at 105000 g for 90 min.

The supernatant was applied to a 0.2 mL HisPur™ Ni-NTA Spin Column, 0.2 mL (Thermo Fisher Scientific) equilibrated with the same buffer. Following one hour of incubation of the closed column under periodical stirring, the column was centrifuged at 1000 rpm for 2 min. The column was washed with multiple subsequent 1 ml portions of the same CHAPS-containing buffer until the optical density of the flow-through at 280 nm decreases below 0.025 OD units. The bound protein was eluted with 500 mM K-Phosphate buffer, 7.4, containing 20% glycerol, 0.5% CHAPS, and 250 mM Imidazole. The detergent was removed with the use of a Bio-Beads

SM-2 resin (Bio-Rad, Hercules, CA, USA) and the protein solution was concentrated to 10 – 20 mg/mL with the use of a Centrisart I MWCO 100 kDa concentrator (Sartorius AG).

2.8. Untargeted proteomics assays

The proteins extracted from Ni-NTA resin were subjected to SDS-PAGE on 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Hercules, CA, USA) using the standard procedure. The Broad Multi Color Pre-Stained Protein Standard from GenScript (Piscataway, NJ) was used for calibration. The gels were stained with Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad) and subjected to fragmentation, as described under Results. The resulting gel fragments were subjected to untargeted proteomic analysis.

The analysis of HLM(LFJ) samples was done in Viktor Zgoda's laboratory using the equipment of "Human Proteome" Core Facilities of the Institute of Biomedical Chemistry (Moscow, Russia). The fragments of the SDS-PAGE slabs were first washed twice with 10% acetic acid and 20% ethanol for 10 min, and then five times with HPLC grade water for 2 min and two times with 40% acetonitrile and 50 mM NH_4HCO_3. After drying with acetonitrile and on air, the gel fragments were digested by trypsin using a previously described protocol [28]. MS analysis was performed with a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Rockwell, IL, USA) as described earlier [28]. Protein identification was performed in Mascot software (Matrix Science, Boston, MA) with decoy [29].

The HLM(LBA) samples were analyzed in the laboratory of Bhagwat Prasad, Department of Pharmaceutical Sciences, Washington State University (Spokane, WA). After washing and drying, the gel fragments were subjected to trypsin digestion using a previously described procedure [22]. MS analysis was carried out using a nanoLC coupled to a Q Exactive HF hybrid quadrupole-orbitrap™ mass spectrometer (Thermo Fisher Scientific, Rockwell, IL, USA) [30].

The obtained raw data were processed using the MaxQuant software (https://maxquant.org) with the built-in search engine Andromeda. Perseus software (https://maxquant.net/perseus/) was used for data visualization and statistical analysis [31].

In both cases protein identification was performed against the complete human proteome provided by Uniprot. Carbamidomethylation of cysteines was set as fixed modification and protein N-terminal acetylation as well as oxidation of methionines was set as variable modification for the peptide search. The false discovery rates (FDR) for peptide and protein identifications were set to 1%.

3. Results

3.1. Modification of CYP2E1 with BPM and BPS.

Incubation of CYP2E1 with BPM in the conditions described under Materials and Methods resulted in incorporation of up to 3 molecules of label per molecule of the enzyme. No increase in the degree of modification or precipitation of denatured protein was observed at increasing the amount of added BPM up to 6 molar equivalents to the protein. Further increase in the molar ratio of BPM added to the protein to 10:1 caused precipitation of the protein and did not result in a noticeable increase in the degree of labeling. Therefore, there are only three cysteine residues per molecule of CYP2E1 that can be modified by BPM without protein unfolding or inactivation. The BPM-crosslinking experiments described below were performed with the protein labeled at the molar ratio varying from 2.4 to 2.7. Interestingly, successful modification of CYP2E1 by BPS required the presence of detergent (Igepal CO-630, 0.2%). Under these conditions, incubation of CYP2E1 with 9 molar equivalents of BPS did not cause any protein precipitation and resulted in incorporation of 7 molecules of the probe per molecule of the protein.

Fig. 1A exemplifies the spectra of UV-Vis absorbance of the purified CYP2E1 protein and its adducts with BPM and BPS. While modification with BPM at 3:1 molar ratio did not cause any noticeable displacement of the spin equilibrium of the heme protein or its conversion into the inactivated P420 state, incorporation of 7 molecules of BPS per CYP2E1 molecule resulted in a moderate (up to 25%) formation of the P420 form of the heme protein, Similar to what was observed with BPM-CYP2E1 adducts, the spin state of the heme protein (~70% of the high-spin state) remained unaffected upon its modification with BPS. These observations suggest that the modification of the protein with BPM and BPS did not cause considerable changes in the protein structure within the limits of the degree of modification used in our experiments.

3.2. Incorporation of modified CYP2E1 into HLM, its photo-activated crosslinking and subsequent isolation from the membranes.

Similar to that was shown earlier for unmodified CYP2E1[25], incubation of BPM- and BPS-labeled protein with HLM at 1:1 molar ratio of the added CYP2E1 to endogenous microsomal P450 content resulted in incorporation of 70 - 80% of the added protein. After the light exposure of the bait-containing microsomes and solubilization of the membranes with detergent (Igepal CO-630, 0.5%), the extracted CYP2E1 binds quantitatively to Ni-NTA resin. Washing the resin with 30 - 35 column volumes of the CHAPS-containing buffer (see Matrials and Methods) decreased the protein absorbance band in the flow-through from the initial 1.2 to approximately 0.025 OD units. Eluting the bound protein with 0.25 M imidazole allowed recovering labeled CYP2E1 in the amount of up to 50% of that taken for the experiment. The UV/Vis absorbance spectrum of the eluate (Fig. 1, inset) indicated the presence of considerable amounts of crosslinked or unspecifically bound proteins. Consequently, SDS-PAGE assays revealed several noticeable bands that correspond to the proteins with molecular masses different from that of CYP2E1 (Fig. 2). As seen from Fig 2, the pattern of the bands observed in the control experiment

with unlabeled CYP2E1 reveals no obvious difference from that seen with the benzophenone-activated CYP2E1 suggesting that both samples may contain unspecifically-bound proteins.

3.4. Identification of CYP2E1-crosslinked proteins with untargeted proteomics

To identify potential crosslinks of the benzophenone-activated CYP2E1 with other proteins, the fragments of the SDS-PAGE slabs corresponding to the molecular masses equal or higher than that of CYP2E1 (57 kDA) were subjected to untargeted proteomics assays. The scheme of fragmentation of the SDS-PAGE slabs for this analysis is illustrated in Fig. 2.

The proteomics analysis of the SDS-PAGE fragments revealed the presence of various microsomal proteins, of whose the most abundant were CYP2E1, CES1 (liver carboxylesterase 1) and P4HB (protein disulfide isomerase). According to the intensity of bands of the found peptides, these three proteins contribute to over 60% of all proteins found in SDS-PAGE lanes. In order to detect potential crosslinks of activated CYP2E1 with cytochromes P450 and other drug-metabolizing enzymes (DMEs), we analyzed the apparent abundances of these proteins in gel fragments corresponding to their molecular masses and higher. In the case of MASCOT analysis, which was used with HLM(LFJ)-based preparations, the relative abundance of the proteins was estimated from the total number of peptide ion matches. In the case of the experiments with HLM(LBA), which were analyzed with MaxQuant software, the relative abundance was estimated from the total intensity (spectral count) of the respective peptide bands. In both cases, these parameters were normalized by dividing by the sum of the values found for all peptides identified in the band, except those corresponding to potentially contaminants (e.g., keratins and serum albumin).

Our further analysis was focused on detecting potential crosslinks of CYP2E1 with other cytochromes P450, UGTs, and their potential protein-protein interaction partners - cytochrome b5 (CYB5), NADPH-cytochrome P450 reductase (NCPR) and Progesterone Receptor Membrane

Component 1 (PGRMC1). We also analyzed the presence of liver carboxylesterase 1 (CES1) and protein disulfide-isomerase (P4HB), two highly abundant proteins located in the ER lumen, which cannot interact with cytochromes P450 unless the ER membrane is destroyed. These two proteins were used as no-crosslinking references.

The identification of the potentially crosslinked proteins was based on the analysis of their distribution between the different zones of the SDS-PAGE lanes. Theoretically, at no crosslinking taking place, all proteins present in the gel lane must be found in the zones that correspond to their molecular masses. All proteins except NCPR, CYB5, and PGRMC1 have molecular masses in the range of 45-65 kDA and must therefore be found in the Zone 1. However, due to limited resolution of SDS-PAGE, some amounts of these proteins could be also found in the other zones even at no crosslinking taking place. To account for this factor, we normalized the relative abundances calculated as above to the total of the abundances found in all five analyzed zones of the gel. The resulting values characterize the distribution of each particular protein between the zones. At the final step of analysis, we calculated the ratio of these double-normalized values observed in crosslinking experiments to those calculated for the control sample, where non-activated CYP2E1 was subjected to the same procedure as in the experiments with BPM- and BPS-labeled proteins.

The examples of the profiles of crosslinking-to-control ratio calculated in this way are shown in Fig. 3. The contrasting difference of the profiles obtained for CYP3A4, CYP2C9 and CYP2A6 from those calculated for CES1 and P4HB suggests an abundant formation of crosslinked aggregates of the former three proteins with the bait (CYP2E1). As seen from this figure, the averaged crosslinking-to-control ratios for CYP3A4, CYP2C9 and CYP2A6 calculated for the SDS-PAGE zones corresponding to molecular masses >65 kDA are up to two orders of magnitude higher than those observed with CES1 and P4HB, non-crosslinking reference proteins.

Importantly, the results obtained with two different crosslinkers – BPM and BPS – exhibit similar patterns. In both cases the profiles obtained with P450 enzymes display a pronounced maximum in the zone corresponding to the molecular masses of 90 – 130 kDA that suggests a predominant formation of dimeric crosslinks with the bait. Interestingly, the profiles obtained with BPS suggest more extensive formation of trimeric aggregates. This observation is consistent with the higher degree of labeling in BPS-modified CYP2E1 as compared to its BPM-modified counterpart (seven benzophenone groups per protein molecule with BPS, as compared to three in the case of BPM).

Results of calculating crosslinking-to-control ratio for all DMEs found in the gel fragments in all six individual experiments (three experiments with each of the two crosslinking agents) are summarized in Table 1. In further analysis, we identified the proteins that exhibited the crosslinking-to-control ratio higher than 10 in any of the gel zones corresponding to molecular masses higher than that of the respective protein itself (zones 2-5 for P450s and UGTs, zones 3-5 for NCPR and zones 1-5 for cytochrome b_5 and PGRMC1). The proteins matching this criterion in five or six individual experiments were considered as the most probable protein-protein interaction partners of CYP2E1. The list of these apparent interaction partners includes cytochromes P450 2A6, 2C9, 3A4 and 4A11, as well as UGTs 1A6, 1A9, 2B15, 2B17, and 2B4.

4. Discussion.

To our knowledge, this study is the first example of using CXMS technique with a membrane-incorporated protein activated by a photo-sensitive crosslinking reagent. Our results demonstrate the high exploratory power of this new approach. It allowed us to detect interactions of alcohol-inducible CYP2E1 protein with cytochromes P450 2A6, 3A4, 2C9, and 4A11, and UDP-glucuronosyltransferases (UGTs) 1A6, 1A9, 2B4, 2B15, and 2B17.

Detection of multiple CYP2E1-crosslinked DMEs in our CXMS experiments corroborates the premise of a complex network of inter-protein interactions in the human drug-metabolizing ensemble. Identification of CYP3A4 as one of the most prominent interaction partner of CYP2E1 is an a good agreement with our recent observation of a multifold activation of CYP3A4 in both CYP2E1-enriched microsomes and HLM preparations obtained from donors with a history of chronic alcohol exposure[25]. It also agrees with the results of our studies of CYP2E1-CYP3A4 interactions with LRET [32] and homo-FRET[25] techniques. Extensive interactions of CYP2E1 with CYP3A4 and CYP2C9 suggested by our results provide possible explanations for the alcohol-induced increase in the metabolism of diazepam and doxycyclin[17-19], the substrates of CYP3A, or phenytoin, tolbutamide, and warfarin[20-21] metabolized primarily by CYP2C9. CYP2E1 interactions with CYP2A6 suggested by our results may give a clue for increased rate of metabolism of nicotine, a CYP2A6 substrate, in alcohol-dependent smokers [33]. Of particular interest is the observation of the efficient crosslinkinking of CYP2E1 with CYP4A11. This enzyme plays a central role in the synthesis of vasoactive eicosanoids and its interactions with alcohol-inducible CYP2E1 may shed light on the mechanisms of alcohol-induced hypertension.

All four P450 enzymes identified by CXMS experiments as the protein-protein interaction partners of CYP2E1 belong to the list of most abundant P450 species in the ER of liver cells. The fractional content of each of CYP2A6, CYP2D6 and CYP3A4 contribute at least 12% to the total pool of drug-metabolizing P450 species in both HLM(LFJ) and HLM(LBA) [25]. The fractional content of CYP2A11 in human liver is also relatively high, and was estimated to be around 8% [34]. The only drug-metabolizing P450 enzyme other than CYP2E1 with a comparable abundance is CYP1A2, which was not among the top CXMS hits. It is, however, possible that the crosslinks of CYP2E1 with some other interacting P450 species may be missed by our analysis due to the low abundance of these proteins in HLM.

Detection of the CYP2E1 crosslinks with UGTs 1A6, 1A9, 2B4, 2B15, and 2B17 is in-line with multiple reports on physical interactions between cytochromes P450 and UGTs interactions and their functional effects [35-36]. Most of these reports relate to the formation of complexes of UGTs 1A and 2B with CYP3A4 [37-42], although the UGTs interactions with CYP1A1 [43], and CYP1A2 [44] were also detected. To our knowledge, the present study is the first that suggests the interactions between UGTs with CYP2E1. Validation of these interactions in a direct investigation and evaluation of their possible functional consequences may provide insight onto the effects of alcohol exposure on the metabolism of drug substrates of UGTs, such as morphine and other opioids.

In total, our results demonstrate the high exploratory power of the proposed CXMS strategy and corroborate the concept of tight functional integration in the human drug-metabolizing ensemble through protein-protein interactions of the constituting enzymes. Further development of the proposed approach and its application for studying the interactome of other P450 enzymes will help to elucidate the entire network of protein-protein interactions in the drug-metabolizing ensemble and understand the mechanisms of its integration into a multienzyme system.

Declaration of competing interest

The authors declare that there are no competing interests associated with the manuscript.

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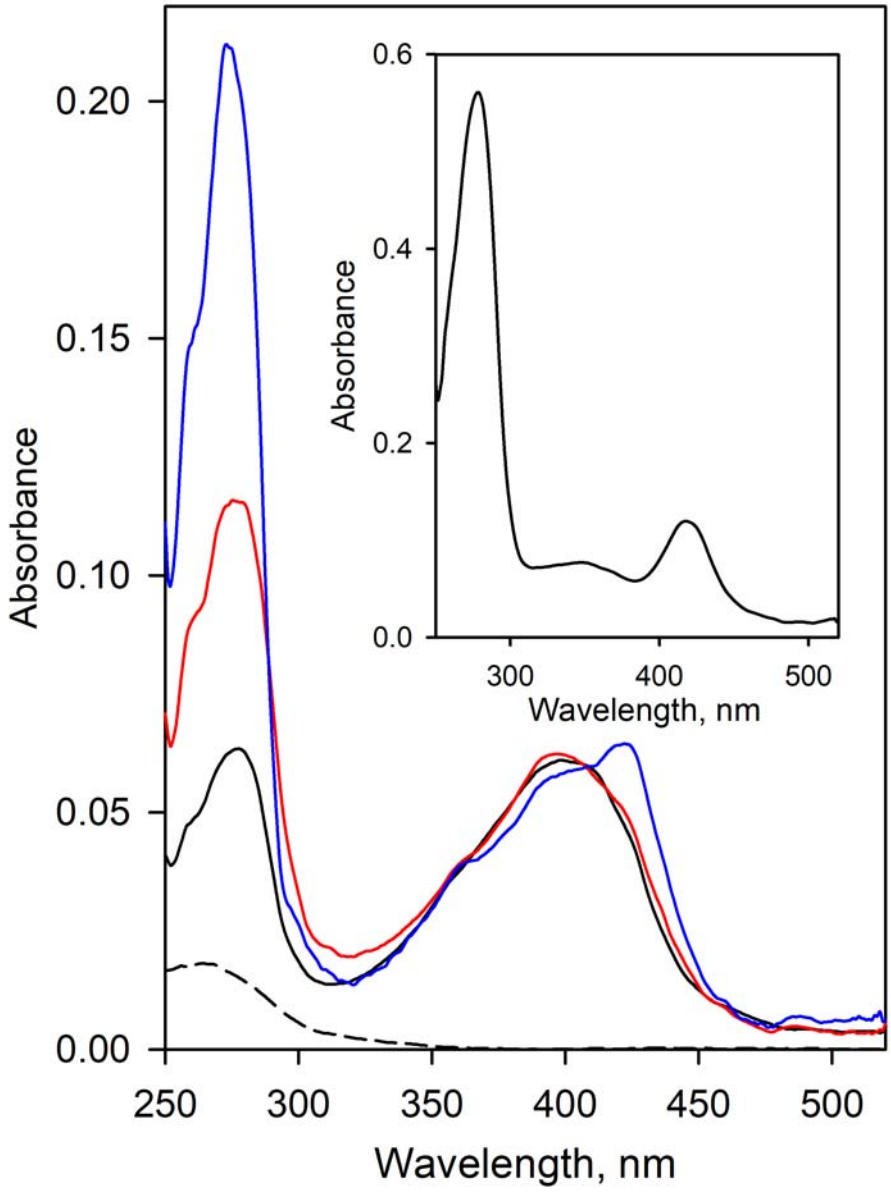

Fig. 1. **Modification of CYP2E1 with BPM and BPS.** The graph shows the absorbance spectra of purified CYP2E1 (black solid line), benzophenone-4-maleimide (black dashed line), and CYP2E1 modified with BPM (red) and BPS (blue). The inset shows the spectrum of the BPM-modified protein eluted from the Ni-NTA resin after the crosslinking experiment. The spectrum was taken in the presence of 0.25M inidazole. All spectra were normalized to correspond to the heme protein concentration of 1 μM.

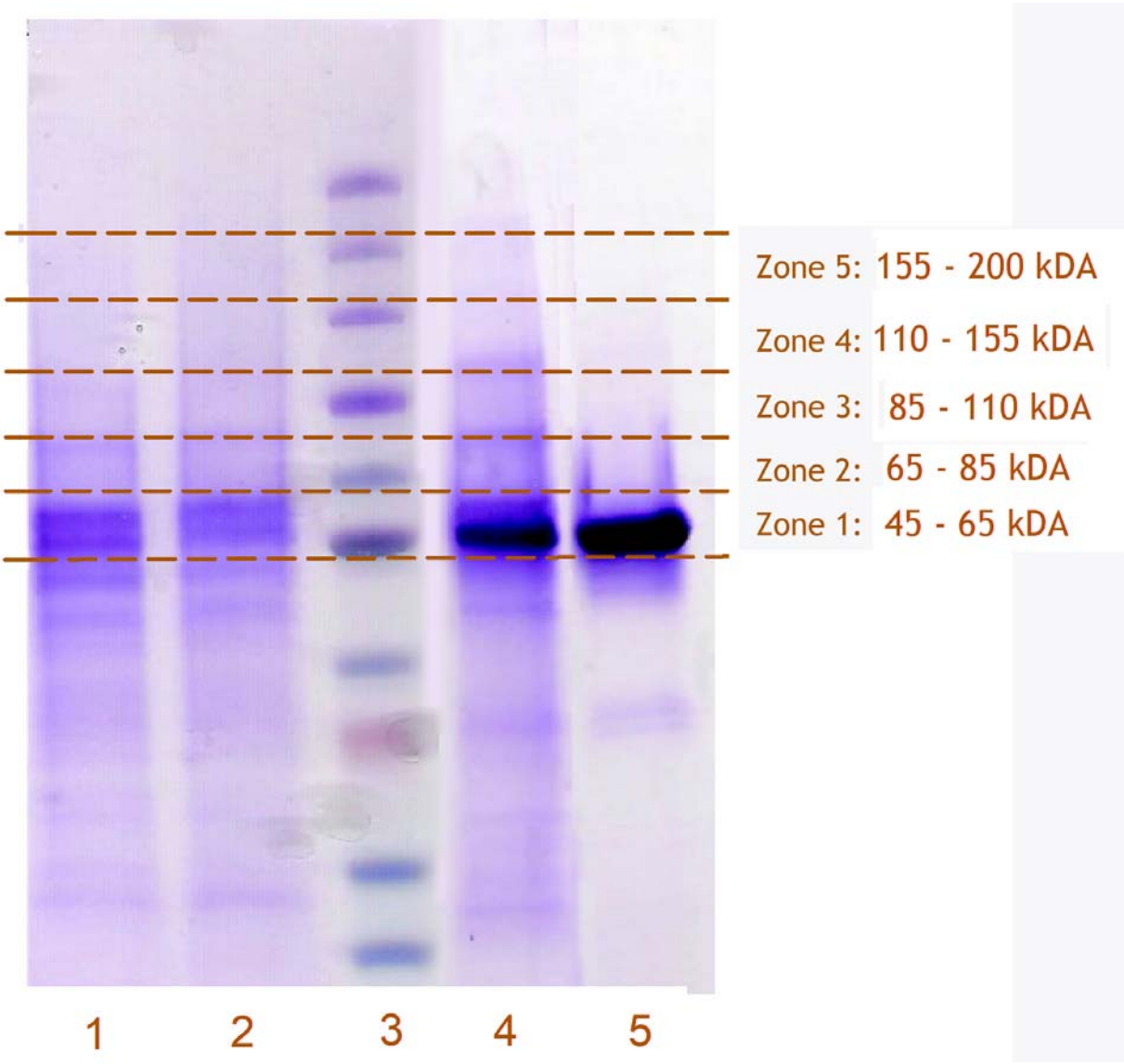

Fig. 2. The scheme of fragmentation of SDS-PAGE slabs for MS/MS analysis. The SDS-PAGE slab shown in the figure exemplifies the lanes loaded with proteins obtained in crosslinking experiments with HLM(LFJ) using BPM-CYP2E1 (1) and BPS-CYP2E1 (2) as a bait protein, and a control experiment with the same microsomes subjected to incorporation and isolation of unlabeled CYP2E1 (4),. Lanes 3 and 5 correspond to the calibrating protein ladder (5, 15, 30, 35, 50, 65, 95, 130, 175 and 270 kDA) and the purified CYP2E1 protein, respectively.

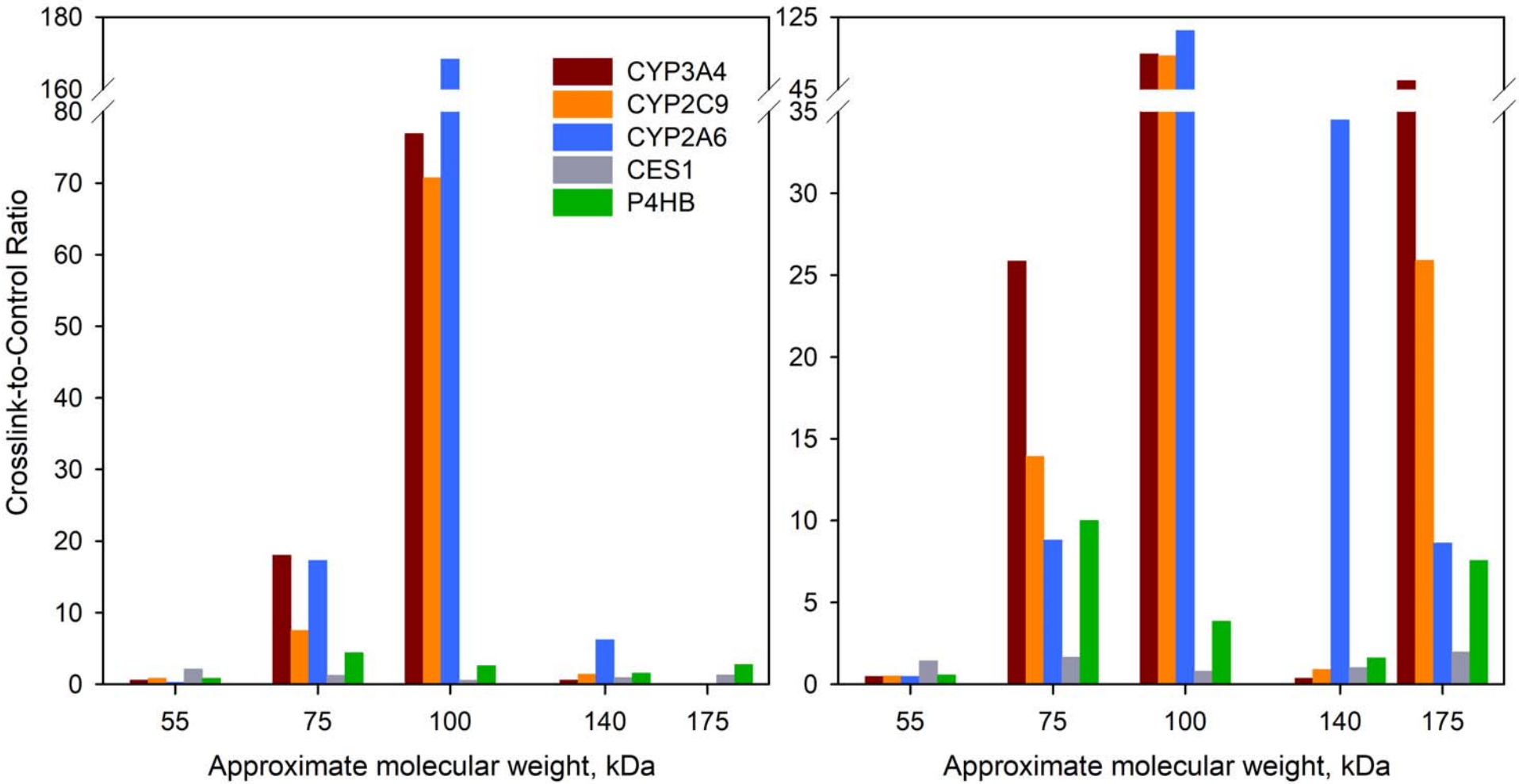

Fig 3. **Distribution of potentially cross-linked proteins between the individual bands of the SDS-PAGE lanes obtained in the experiments with BPM-CYP2E1 (A) and BPS-CYP2E1(B).** The data shown in the graph represent the averages of three individual experiments with HLM(LFJ) and HLM(LBA) summarized in Table 1. The Y-axis of the plot corresponds to the ratio of normalized apparent protein abundance in the cross-linked sample to that observed in the control. Ordinate of the graph shows the approximate averaged molecular weights of all proteins found in each band.

Table 1. Results of six individual CXMS experiments*

Protein	Crosslinkimg with BPM															Crosslinking with BPS															Total positives
	HLM(LFJ), assay 1					HLM(LFJ), assay 2					HLM(LBA)					HLM(LFJ), assay 1					HLM(LFJ), assay 2					HLM(LBA)					
	55 kDA [a]	75 kDA	100 kDA	140 kDA	180 kDA	55 kDA	75 kDA	100 kDA	140 kDA	180 kDA	55 kDA	75 kDA	100 kDA	140 kDA	180 kDA	55 kDA	75 kDA	100 kDA	140 kDA	180 kDA	55 kDA	75 kDA	100 kDA	140 kDA	180 kDA	55 kDA	75 kDA	100 kDA	140 kDA	180 kDA	
CYP2E1	1.3	1.8	0.3	1.9	3.0	0.6	0.8	1.1	1.6	1.9	0.9	1.7	0.5	1.0	0.5	1.5	1.7	0.3	1.4	3.1	0.5	0.8	1.3	1.6	2.0	0.5	4.4	0.7	0.8	0.4	0
CYP1A2	0.7	0.8	245	0	0	0.7	1.8	1.5	0	0	0.7	∝	0	0	0	1.1	0.7	0	0	0	0.5	2.5	2.0	0	0	0	∝	0	0	0	3
CYP2A6 [b]	**0.4**	**0.7**	**486**	**0.6**	**0**	**0.3**	**0.9**	**1.9**	**0**	**0**	**0.1**	**50**	**17.6**	**18.0**	**0**	**0.7**	**0.6**	**306**	**1.0**	**0**	**0.6**	**1.0**	**1.1**	**83**	**0**	**0.1**	**25**	**23**	**19**	**26**	**5**
CYP2B6	0.6	206	207	0	0	82	294	624	0	0	0	0	0	0	0	1.0	0	0	0	0	125	260	∝	0	0	0	0	0	0	0	3
CYP2C18	∝	0.9	0	0	0	0	0.4	554	0	0	0	0	0	0	0	∝	0	0	0	0	0	0	∝	0	0	0	0	0	0	0	2
CYP2C19	0.7	1.9	147	0.5	105	0.8	0.7	2.4	107	0	0	0	0	0	0	0.7	3.0	279	0.3	0	0.4	1.0	2.9	167	0	0	0	0	0	0	4
CYP2C8	1.6	1.4	1.0	0.3	0.7	0.8	0.5	3.2	1.0	0.9	0.4	13	6.6	0	0	1.9	0.6	1.0	0.4	1.9	0.5	0.4	3.4	0.8	3.5	0.1	8.5	4.7	3	8.3	1
CYP2C9	**1.0**	**2.2**	**206**	**0.3**	**0**	**0.8**	**1.5**	**2.0**	**0.6**	**0**	**0.6**	**19**	**4.7**	**3.2**	**0**	**0.8**	**2.9**	**238**	**0.3**	**0**	**0.4**	**2.7**	**2.1**	**0.9**	**73**	**0.3**	**36**	**6.8**	**2**	**4.6**	**5**
CYP2D6	1.2	0.5	0	0	0	0.3	245	432	0	0	1.0	0	0	0	0	1.4	0.1	0	0	0	0.3	334	345	0	0	0	∝	0	0	0	3
CYP3A4[c]	**0.7**	**1.9**	**226**	**0.6**	**0**	**0.6**	**1.9**	**1.2**	**1.0**	**0**	**0.5**	**50**	**3.3**	**0**	**0**	**0.9**	**1.5**	**244**	**0.4**	**0.4**	**0.4**	**1.8**	**1.2**	**0.8**	**150**	**0**	**74**	**7.6**	**0**	**14**	**5**
CYP3A5	1.2	119	150	0.7	0	1.0	288	0.7	0.8	0	0	0	0	0	0	2.0	0	194	0.2	0	0	413	1.9	0.7	0	0	0	0	0	0	4
CYP4A11[d]	**0.5**	**1.4**	**257**	**0.5**	**0**	**0.5**	**3.2**	**11**	**0.4**	**0**	**0.6**	**22**	**0**	**0**	**0**	**1.1**	**0.7**	**51**	**0**	**0**	**0.3**	**2.7**	**17**	**0**	**0**	**0.2**	**37**	**2.4**	**0**	**0**	**6**
CYP4F12	0.7	254	91	0	0	0.8	0.6	195	0	0	1.0	0	0	0	0	1.0	0	0	0	0	0.7	1.4	165	0	0	0	0	0	0	0	3
CYP4F2	0.7	1.1	174	1.0	0	0.7	1.4	152	0.8	0	1.2	1.6	0	0	0	1.0	0	213	0.5	0	0.6	1.7	180	0.3	180	1.2	2.1	0	0	0	4
CYP4F8	∝	0.2	229	0	0	0.8	249	0	0	0	0	0	0	0	0	∝	0	0	0	0	0.6	239	188	0	0	0	∝	0	0	0	4
CYB5A	0	1.0	0	0	0	229	∝	226	0	0	0	0	0	0	0	127	0.9	0	0	0	220	306	316	0	158	0	0	0	0	0	2
NCPR	1.4	1.2	0.4	0.7	0	2.2	0.7	1.7	1.7	0	8.5	336	2.0	0	0	2.1	1.0	0.5	1.3	0	3.4	0.6	1.5	1.7	39	18	56	36	0	1	2
PGRMC1	0	1.0	0	0	0	0	1.0	0	0	0	0	0	0	0	0	225	0.8	0	0	0	385	0.5	140	0	0	0	0	0	0	0	2
UGT1A1	1.0	1.0	153	0.6	0	0.8	1.6	1.2	1.0	0	1.0	0	0	0	0	0.5	1.4	367	0.5	0	0.6	1.0	1.1	0.8	175	0	∝	0	0	0	4
UGT1A3	0.9	0.7	178	0	0	0.6	364	0	0	0	1.0	0	0	0	0	1.3	0	0	0	0	0.5	203	325	0	0	0	0	0	0	0	3
UGT 1A4 [e]	0.9	0.8	133	0.5	76	0.6	1.1	1.6	1.8	0	1.0	0	0	0	0	0.6	1.4	370	0.3	0	0.7	0.9	2.0	1.0	97	0.5	47	0	0	0	4
UGT 1A6	**0.9**	**0.7**	**157**	**0.5**	**72**	**0.4**	**2.2**	**2.6**	**1.2**	**50**	**0.7**	**∝**	**0**	**2.0**	**0**	**0.7**	**1.2**	**203**	**0.6**	**0**	**0.6**	**2.2**	**2.1**	**0.8**	**89**	**0.6**	**21**	**6.0**	**4**	**5.3**	**6**
UGT 1A9[f]	**0.7**	**110**	**132**	**0.9**	**0**	**0.4**	**236**	**246**	**111**	**0**	**0.5**	**∝**	**5.3**	**1.0**	**0**	**0.6**	**220**	**214**	**0.5**	**0**	**0.4**	**218**	**312**	**92**	**0**	**0.3**	**38**	**5.9**	**1**	**15**	**6**
UGT 2A1	0.7	115	216	0	0	1.2	0	0.7	0	0	0	0	0	0	0	1.0	0	0	0	0	1.0	0	1.0	0	0	0	0	0	0	0	1
UGT2A3	1.0	0	0	0	0	1.0	0	0	0	0	1.0	0	0	0	0	1.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
UGT 2B10	0.5	1.6	223	1.2	1.1	0.5	1.4	1.2	1.9	3.7	0.6	∝	0	0	0	0.6	1.6	112	0.9	1.9	0.4	1.0	1.2	1.3	7.5	0	∝	0	0	0	4
UGT 2B11	0.4	0.8	268	0.9	102	0.6	0.9	0.8	131	137	0	0	0	0	0	1.4	1.5	0	0	0	0.5	0.4	0.8	0	428	0	0	0	0	0	3
UGT 2B15	**0.5**	**0.6**	**215**	**1.4**	**2.5**	**0.5**	**2.5**	**0.6**	**1.5**	**181**	**0.6**	**∝**	**0**	**0**	**0**	**0.8**	**1.0**	**226**	**0.7**	**0**	**0.5**	**2.5**	**0.7**	**0.6**	**287**	**0**	**0**	**0**	**0**	**0**	**5**
UGT 2B17	**1.0**	**1.2**	**249**	**0**	**1.4**	**0.5**	**1.1**	**0.8**	**1.4**	**195**	**0.5**	**27**	**8.3**	**6.5**	**0**	**1.1**	**1.3**	**267**	**0.4**	**0**	**0.5**	**1.1**	**0.9**	**0.8**	**256**	**0**	**45**	**6.8**	**13**	**15**	**6**
UGT 2B28	0.6	1.4	335	0	1.9	0.6	0.8	1.0	1.2	188	0	0	0	0	0	1.5	0.7	120	0.2	0	0.3	0.9	1.4	0.2	437	0	0	0	0	0	4
UGT 2B4	**0.5**	**1.1**	**287**	**0.9**	**0.8**	**0.6**	**2.2**	**0.8**	**1.7**	**120**	**0.7**	**7.7**	**1.7**	**8.1**	**0**	**0.8**	**1.0**	**217**	**0.6**	**0.8**	**0.4**	**1.6**	**1.0**	**1.2**	**237**	**0.4**	**18**	**0**	**18**	**0**	**5**
UGT 2B7	0.5	0.7	212	1.0	1.7	0.6	1.3	1.1	1.1	3.2	0.7	14	3.8	7.6	0	0.5	0.7	230	0.9	1.5	0.4	1.1	1.1	1.2	4.9	0.1	38	8.6	9.2	17	4
CES1	1.9	1.7	0.2	1.4	2.3	1.0	0.8	0.8	0.9	1.6	3.5	1.2	0.7	0.6	0.1	1.2	1.5	0.3	1.5	3.1	1.0	0.8	1.0	1.1	1.1	2.1	2.7	1.1	0.4	1.7	0
P4HB	1.0	1.4	0.3	2.5	3.3	0.7	1.0	1.3	1.3	2.4	0.8	11	6.3	0.8	2.6	0.7	1.3	0.4	2.2	5.3	0.5	0.8	1.5	1.8	2.7	0.5	28	9.7	0.8	15	2

*The values shown in the table represent the ratios of the normalized apparent abundances of the proteins in the SDS-PAGE bands of cross-linked proteins to those observed in the control with unlabeled CYP2E1. The infinity sign (∞) indicates the cases where no protein was found in the control, while it was present in the cross-linked sample. Zeros correspond to the cases when no protein found in either the cross-linked samples only, or in both the crosslinked sample and the contol. The ratios of 10 and higher observed in the bands corresponding to molecular masses of 75 kDA and higher were considered as indicative of cross-linking with the bait. The exception is cytochrome b_5 (CYB5), for which the ratio observed in the 55 kDA band was also taken into account, and NCPR, for which only the bands starting at 100 kDA were considered. The proteins that meet this criterion in five or six experiments were selected as the most probable protein-protein interaction partners of CYP2E1. The lines corresponding to these proteins are shown in bold.

[a] This row shows the approximate average molecular weight corresponding to the SDS-PAGE bands

[b] This row corresponds to both CYP2A6 and CYP2A7, which could not be reliably resolved in MS/MS assays.

[c] This row corresponds to both CYP3A4 and CYP3A7

[d] This row corresponds to both CYP4A11 and CYP4A22.

[e] This row corresponds to both UGT 1A4 and UGT 1A5.

[f] This row corresponds to the total of UGT 1A7, 1A8, 1A9, and 1A10.

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