

## Photo-cytochrome b<sub>5</sub> – A New Tool to Study the Cytochrome P450 Electron-transport Chain

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To study the enigmatic role of cytochrome b<sub>5</sub> in a cytochrome P450 monooxygenase multienzyme system, we expressed and purified a photoreactive analogue of cytochrome b<sub>5</sub>, having methionine residues substituted with photolabile diazirine derivatives (photo-cyt b<sub>5</sub>). An equimolar mixture of cytochrome P450 (CYP) 2B4 and photo-cyt b<sub>5</sub> was photolyzed and products separated using SDS-PAGE. Several oligomers composed of CYP2B4 and cyt b<sub>5</sub> (MALDI-TOF/TOF mass spectrometry) in 1:1, 1:2, and 2:1 molar ratios have been found and their covalent nature confirmed by MALDI-TOF/TOF mass spectrometry of chymotryptic peptides. The amount of oligomers formed was markedly increased by the presence of the CYP2B4 substrate, diamantane, showing that the substrate binding increases the affinity between cytochromes P450 and b<sub>5</sub>.

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**Keywords:** Electron-transport chain; Photo-methionine; Cytochrome b<sub>5</sub>; Cytochrome P450 2B4; Mass Spectrometry; Cross-linking

### 1. INTRODUCTION

The mammalian mixed function oxidase system (MFO) localized in the membrane of endoplasmic reticulum is composed of cytochrome P450 (CYP), flavoprotein NADPH:cytochrome P450 reductase (CYPOR), and cytochrome b<sub>5</sub> (cyt b<sub>5</sub>) accompanied with its NADH:cytochrome b<sub>5</sub> reductase. Via the activation of molecular oxygen this multienzyme system catalyzes the monooxygenation of endogenous compounds as well as foreign chemicals (xenobiotics) [1]. The oxygen is activated in the active center of CYPs by two electrons transferred from NADPH and/or

NADH by means of CYPOR and cyt b<sub>5</sub>, respectively. While CYPOR is an essential constituent of the electron transport chain towards CYP, the role of cyt b<sub>5</sub> is quite enigmatic. The catalytic activity of the CYP monooxygenase system might be altered by the presence of cyt b<sub>5</sub>. Depending on the CYP form, cyt b<sub>5</sub> may inhibit or stimulate metabolism of a particular substrate or even change the pattern of metabolites formed [2-4]. The mechanism underlying this effect has not been fully explained yet. The inhibition of CYP-mediated catalytic activity by cyt b<sub>5</sub> is attributed to the competition of cyt b<sub>5</sub> and CYPOR for the binding site on CYP [5]. The transfer of electrons from cyt b<sub>5</sub> to CYP, which can be directly monitored by electrochemical techniques [6-8], may explain cyt b<sub>5</sub> stimulatory effects. Moreover, experiments with apo-cyt b<sub>5</sub> [9] suggest that cyt b<sub>5</sub> may induce conformational changes of the CYP active site resulting in the facilitation of a substrate binding and/or its metabolism [3,4]. As cyt b<sub>5</sub> may significantly affect the activity of the MFO system, which is participating in the metabolism of drugs and activation of carcinogens [10], it is important to explain the molecular basis of cyt b<sub>5</sub> role. The present study deals with a novel approach of chemical cross-linking, which is designed to elucidate the protein-protein interactions within MFO system in the membrane environment.

## 2. MATERIAL AND METHODS

### 2.1 Expression of cytochrome b<sub>5</sub> with incorporated photo-methionine

To prepare cytochrome b<sub>5</sub> with incorporated photo-methionine (pMet), the heterologous expression in *E. coli* as described by us was utilized [11]. Briefly, the gene for rabbit cyt b<sub>5</sub> was constructed from synthetic oligonucleotides using polymerase chain reaction, cloned into pUC19 plasmid and amplified in DH5a cells. The sequence coding for cyt b<sub>5</sub> was cleaved from pUC19 by NdeI and XhoI restriction endonucleases and subcloned into the expression vector pET22b. This vector was used to transform *E. coli* BL-21 (DE3) Gold cells. Our original protocol of cyt b<sub>5</sub> production was further optimized in terms of the photo-methionine concentration, timing and length of individual steps to assure the maximal incorporation of photo-methionine into cyt b<sub>5</sub>. Transformed bacteria were cultivated in LB medium at 37 °C to reach an optical density A<sub>600</sub> ~ 0.6. After washing with PBS, bacteria were transferred into Dulbecco's Modified Eagle's Limiting Medium (minus L-leucine and L-methionine (DMEM-LM) and after 0-20 min L-Photo-Methionine (L-2-amino-5,5-azihexanoic acid, Pierce Biotechnology, USA) and D,L-leucine were added to final concentration 0.2-2.0 mM and 1.6 mM, respectively. Prior to the induction of cyt b<sub>5</sub> expression with isopropyl β-D-1-thiogalactopyranoside (1 mM), 5-aminolevulinic acid hydrochloride (0.5 mM) was added. Thereafter, the incorporation of pMet into cyt b<sub>5</sub> (photo-cyt b<sub>5</sub>) was monitored by mass spectrometry (MALDI-TOF) for 4 hrs. For that purpose the relative intensities of m/z peaks for chymotryptic peptides containing pMet and a natural Met residue (m/z difference 20 Da) were compared.

### 2.2 Purification of photo-cytochrome b<sub>5</sub>

The expressed photo-cyt b<sub>5</sub> was purified from 1 hr cultivation (6 x 30 ml DMEM-LM supplemented with photo-Met and Leu) according to the procedure described earlier [11]. Briefly, bacterial cells were sonicated (8 x 2.5 min, 40 W) in 10 mM potassium phosphate buffer pH 7.7,

containing 20% glycerol, 1 mM EDTA and protease inhibitors (cOmplete ULTRA Tablets, Roche Applied Science, Germany) under a successive cooling on ice. After removal of cell debris (centrifugation at 3000g for 15 min at 4 °C) the membrane fraction of bacteria was collected by ultracentrifugation at 60.000g for 75 min at 4 °C. The resuspended membrane sediment (in 20 mM potassium phosphate buffer pH 7.7, containing 20% glycerol, 1 mM EDTA and protease inhibitors) was solubilized by the mixture of detergents, Brij 35 and sodium cholate (in the ratio 1:1), to reach 1 mg of each detergent per mg of proteins. The mixture was stirred for 2 hrs at 4 °C and insoluble residue was removed by ultracentrifugation at 60.000g for 75 min at 4 °C. Solubilized photo-cyt  $b_5$  was purified on a DEAE Sepharose CL-6B column (1.3 x 15 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, 20% glycerol and 0.6% Brij. After washing the column, bound photo-cyt  $b_5$  was eluted using a linear gradient of KCl (0–400 mM) in the equilibration buffer. The elution was monitored spectrophotometrically at 280 and 413 nm and by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) [12], using 15% separating gel. Pooled fractions were concentrated by ultrafiltration on a PM-10 membrane (Millipore), dialyzed against equilibration buffer and loaded onto a second DEAE Sepharose CL-6B column. The second chromatography was carried out as the first one except of the substitution of Brij with sodium cholate (0.1%). Finally, from the pooled and concentrated fractions the detergent was dialyzed out (50 mM potassium phosphate, 20% glycerol, pH 7.7) and the final photo-cyt  $b_5$  preparation was stored at -80 °C. The incorporation of pMet was verified by MALDI-TOF analysis of chymotryptic peptides.

### 2.3 Photo-cross-linking experiment

The CYP2B4 enzyme was isolated from phenobarbital-induced rabbit liver microsomes as described elsewhere [13]. For a photolysis the mixture of CYP2B4 (2  $\mu$ M) and photo-cyt  $b_5$  (2  $\mu$ M) reconstituted in DLPC lipids (300  $\mu$ M) was prepared. A thin film of DLPC prepared on a glass tube by evaporation of its chloroform solution was suspended in PBS under a successive sonication. After the addition of both enzymes the resulting DPLC suspension was sonicated again and then let it sit for 15 min on ice. To investigate the effect of a CYP2B4 substrate on the CYP2B4-cyt  $b_5$  binding, diamantane (50  $\mu$ M) was added to the reaction mixture. Photo-cyt  $b_5$  in a quartz tube (100  $\mu$ l) was activated by 3 min UV-irradiation (photolyser Oriel equipped with Hg-Arc lamp emitting 254 nm). Immediately after the irradiation reduced glutathione (150  $\mu$ M) was added. Components of the reaction mixture were separated by SDS-PAGE, the formed cross-links (having a molecular weight higher than CYP) were excised from the gel and digested with chymotrypsin as follows: the gels were chopped into small cubes (approx. 1 mm<sup>3</sup>), destained with 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% (v/v) acetonitrile. After complete destaining under sonication, the gel pieces were washed with water, shrunk by dehydration with acetonitrile and re-swollen again in water. Further, the gel was partly dried using a SpeedVac concentrator and then mixed with a cleavage buffer containing 0.05 M 4-ethylmorpholine acetate, 10% (v/v) acetonitrile and 50 ng/ $\mu$ l sequencing grade chymotrypsin (Hoffmann-La Roche Ltd., Switzerland). Digestion was carried out at 37°C overnight. The resulting peptides were extracted with 40% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid and analyzed by mass spectrometry. The peptides were directly loaded on the target, allowed to dry at

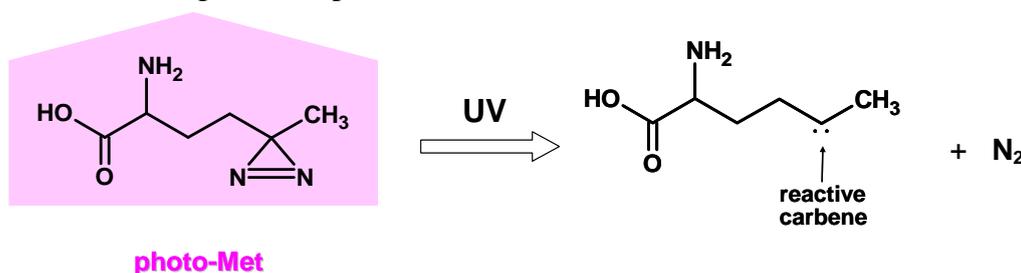
ambient temperature, and over-laid with 25 mg/ml solution of 2,5-dihydroxybenzoic acid in 50% (v/v) acetonitrile, containing 0.1% (v/v) TFA. The positive mass spectra were measured on a matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF/TOF) mass spectrometer ultraFLEX III (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra collected in reflectron mode were calibrated externally using a monoisotopic  $[M+H]^+$  ion of peptide standards PepMix I (Bruker-Daltonics, Bremen, Germany).

#### 2.4 Analytical methods

Protein concentrations were determined by the BCA method [14]. The concentration of cytochrome P450 was estimated according to method by Omura and Sato [15] based on the complex of reduced CYP with CO.

### 3. RESULTS AND DISCUSSION

To study the role of cyt  $b_5$  in a cytochrome P450 multienzyme electron transport chain, the analogue of cyt  $b_5$  containing a photolabile amino acid derivative (photo-Met) instead of methionine was designed. Because of the similarity of photo-Met with a natural Met the amino acid analogue could be incorporated into proteins during the protein synthesis [16]. The structure of this diazirine-compound mimicking methionine and its photolytic activation are shown in Figure 1. Amino acid methionine was selected since it is present only at three positions in the cyt  $b_5$  sequence (see Fig. 2); in two cases the Met residues are localized in a short hydrophobic C-terminal membrane anchor (predicted transmembrane helix spans Asn<sup>109</sup> – Met<sup>131</sup>). N-terminal Met was not considered as this residue is missing in cyt  $b_5$  expressed in *E. coli* [11]. The position of Met residues makes photo-cyt  $b_5$  to be useful for cross-linking of MFO proteins in the membrane environment.

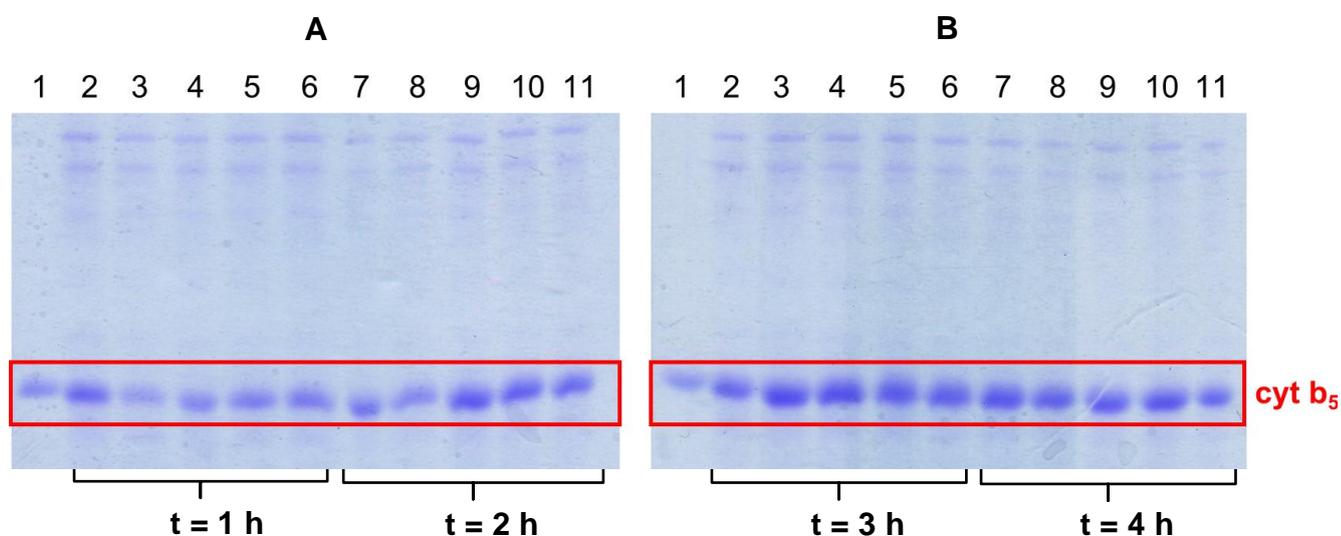


**Figure 1.** UV-light photolysis of diazirine analogue of methionine (photo-Met) resulting in formation of highly reactive carbene.

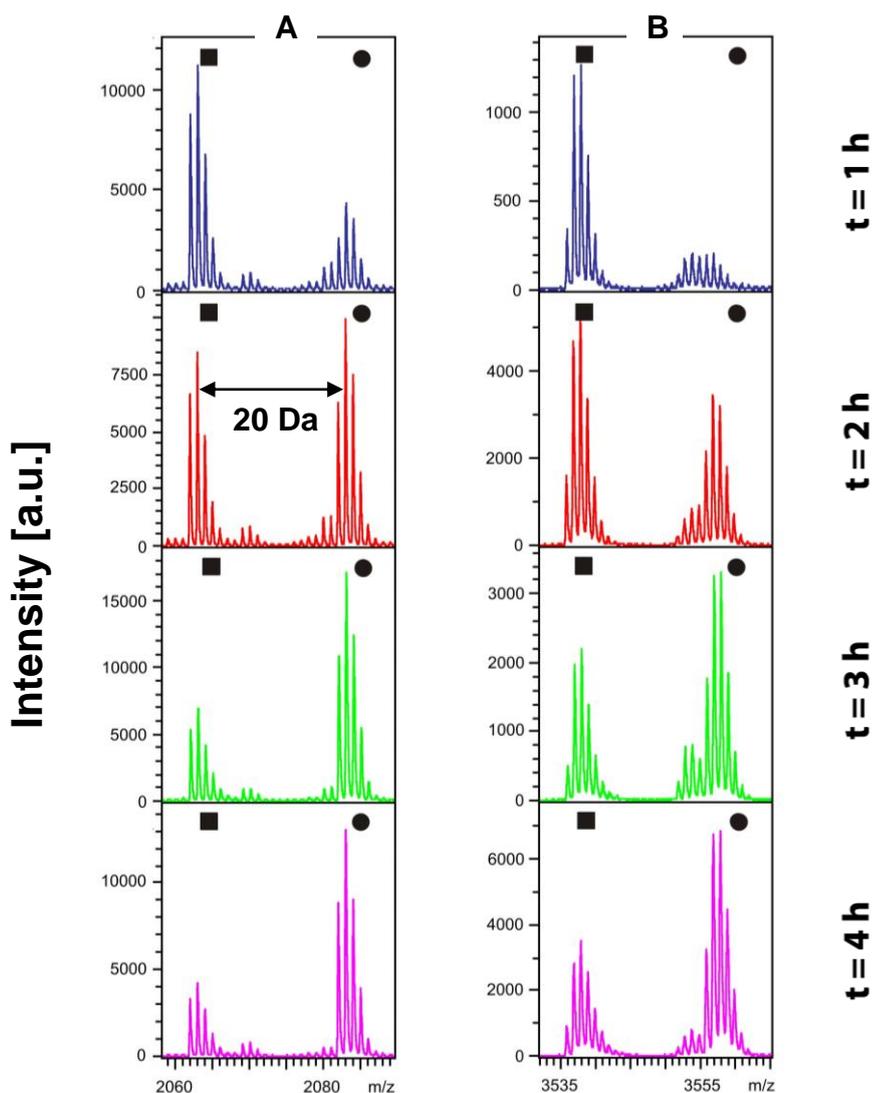
(M)AAQSDKDVKYITLLEEIKKHNSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQAGGDATENFEDVG  
 HSTDARELSKTFIIGELHPDDRSLKSKP**M**ETLITVDSNSSWWTNWVIPAISALIVAL**M**YRL**M**ADD<sub>COOH</sub>  
 96 126 131

**Figure 2.** Amino acid sequence of rabbit recombinant photo-cytochrome  $b_5$  with highlighted positions, in which methionine residues were substituted for photoactivatable analogues. N-terminal Met (in parenthesis) is usually missing in bacteria expressed cytochrome  $b_5$ .

To elaborate an optimal experimental protocol assuring a maximal incorporation of an externally added amino acid to cyt  $b_5$  proteins, the expression of cyt  $b_5$  was first carried out with deuterium labeled Met, L-methionine-methyl-d<sub>3</sub>. As judged from mass spectrometry analysis the produced cyt  $b_5$  contained all Met residues (except of missing N-terminal one) entirely labeled with deuterium (data not shown). In these pilot experiments the optimal optical density of bacterial inoculum ( $A_{600} \sim 1.0$ ) and production time (4 h) were found. Under these conditions, the time dependence of photo-Met incorporation into cyt  $b_5$  under increasing concentrations of photo-Met (0.2 - 2.0 mM) was examined. The cyt  $b_5$  production, monitored as cyt  $b_5$  protein band intensity on SDS-PAGE (see Fig. 3), was apparently time-dependent, but the amount of cyt  $b_5$  was not markedly affected by the concentration of photo-Met. MALDI-TOF analysis revealed only a low Met substitution with photo-Met. This finding suggests that a protein synthesis machinery of *E. coli* prefers to re-cycle Met from bacteria cells instead of using a non-natural amino acid externally added. Two ways how to increase the photo-Met incorporation were suggested and tested. First, the inoculum was prepared of bacterial cells directly from plates and the bacteria were allowed to grow only to optical density of 0.6. This arrangement was aimed at reducing the amounts of old and dead cells. Second, in order to lower Met pool, the washed bacteria transferred from LB to DMEM-LM medium were incubated for at least 20 min before the addition of photo-Met. Under these newly adjusted conditions, photo-cyt  $b_5$  started to be produced and thus the expression protocol could be further optimized. The time-course of photo-Met incorporation was precisely monitored by mass spectrometry. As a representative figure of the whole process we present the photo-Met incorporation into a sequence position of 96. Two chymotryptic peptides containing a single Met<sup>96</sup> (Ser<sup>93</sup> - Trp<sup>110</sup>,  $m/z = 2082.0$ ; Ile<sup>80</sup> - Trp<sup>110</sup>,  $m/z = 3555.8$ ) were selected for the analysis to show that regardless the peptide length their MS data are comparable.

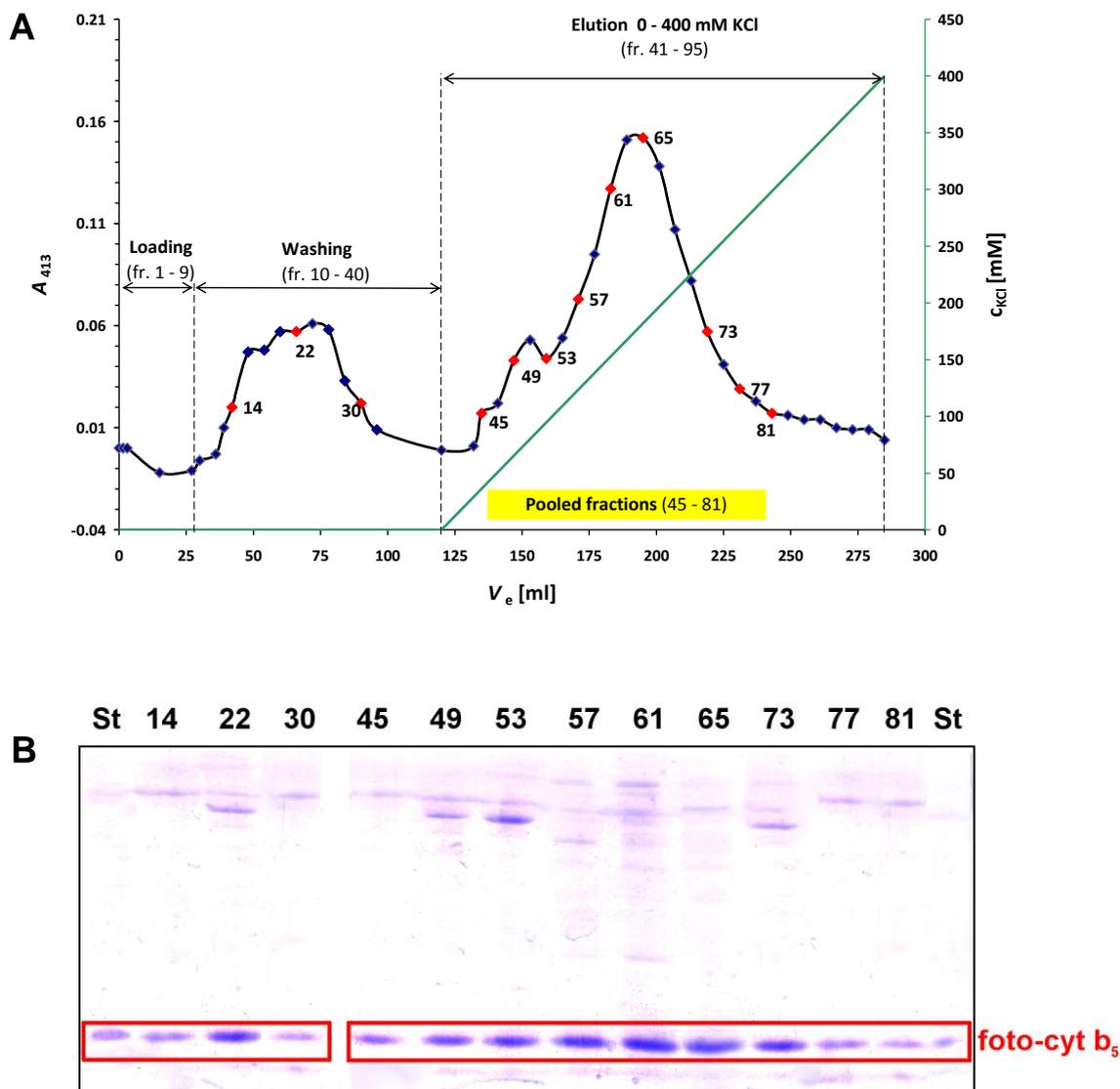


**Figure 3.** SDS-PAGE of photo-cyt  $b_5$  production - time and photo-Met concentration dependence. Extracts from the equal amount of bacterial cells were separated on 15% gel. Panel **A** shows samples from 1 (lines 2-6) and 2 h (lines 7-11), and panel **B** samples from 3 (lines 2-6) and 4 h (lines 7-11) of production. The expression of cyt  $b_5$  at 0.2, 0.5, 1.0, 1.5, and 2.0 mM photo-Met is shown in lines 2+7, 3+8, 4+9, 5+10, and 6+11, respectively. Standards of cyt  $b_5$  were loaded to line 1 in both panels.



**Figure 4.** Mass spectra of selected chymotryptic peptides of cytochrome  $b_5$ . The masses of peptides,  $\text{Ser}^{93}\text{-Trp}^{110}$  and  $\text{Ile}^{80}\text{-Trp}^{110}$ , are shown in panel **A** and **B**, respectively. Peptides containing photo-Met (■) and a natural Met (●) differ by 20 Da because of a degradation of diazirine group of photo-Met upon MS analyses. Relative intensities of corresponding peaks were monitored within the production period 1-4 hrs. The presence of numerous peaks for each peptide reflects naturally occurring isotopes in amino acids.

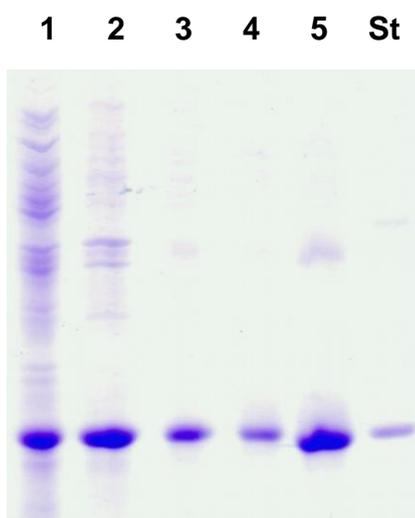
Data presented in Figure 4 show that the highest photo-Met/Met ratio was achieved in 1 h after an IPTG induction. The gradual decline of that ratio occurring later reflects the elevated production of cyt  $b_5$  on expenses of the recycled natural Met. As comparable photo-Met/Met ratios in the expressed cyt  $b_5$  were achieved with 0.5 and 2 mM photo-Met in DMEM-LM medium (data not shown), the 0.5 mM concentration of photo-Met was used for a large-scale photo-cyt  $b_5$  production. Thus, our experimental set-up allowed lowering (4 times) the concentration of photo-Met in DMEM-LM medium compared to data in the literature [16].



**Figure 5.** SDS-PAGE of photo-cyt  $b_5$  purification on DEAE Sepharose CL-6B. Selected fractions of the elution profile labeled with red diamonds (panel **A**) were separated on 15% gel (panel **B**). Fractions 45-81 (in red frame) were pooled for further purification. **St** – refers to lines with cyt  $b_5$  standard loaded.

The expressed photo-cyt  $b_5$  was purified from the solubilized membrane fraction of ultrasound disrupted bacteria [11]. The combination of two detergents, Brij 35 and sodium cholate, instead of Tergitol NP-10 used by Mulrooney and Waskell [17], proved to be effective for releasing of photo-cyt  $b_5$  from membranes. The purification of photo-cyt  $b_5$  on a DEAE Sepharose CL-6B column was followed by measuring  $A_{280}$  and  $A_{413}$ , and by SDS-PAGE of selected fractions (see Fig. 5). A significant part (~ 25%) of the solubilized hemoprotein did not bind to DEAE Sepharose CL-6B and was eluted with washing buffer. Most likely, this flow-through fraction of the photo-cyt  $b_5$  sample contained misfolded protein not interacting with the column. The second DEAE Sepharose CL-6B chromatography was used for removal of Brij 35, while the sodium cholate was dialyzed out from the

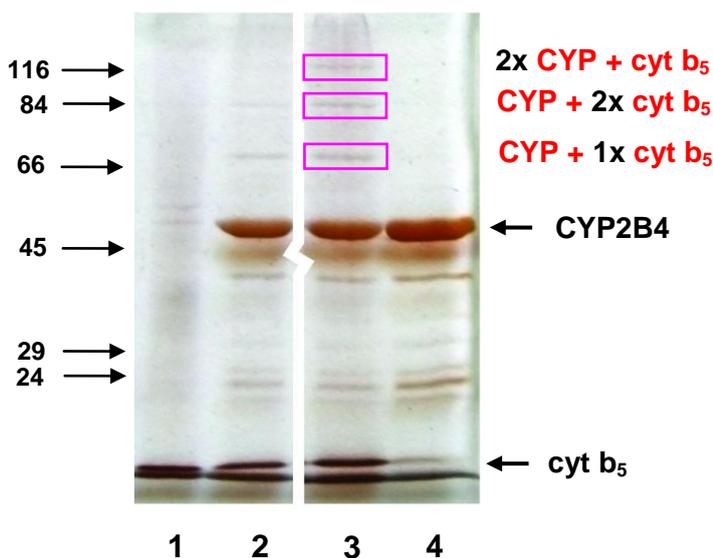
sample of photo-cyt  $b_5$ . SDS-PAGE of samples from individual steps of the purification protocol is shown in Figure 6. Using this procedure an electrophoretically homogeneous sample of cyt  $b_5$  was prepared. The extra band in the final cyt  $b_5$  preparation was identified as a cyt  $b_5$  aggregate (Fig. 6, line 5). The spectroscopy analysis of the final photo-cyt  $b_5$  preparation revealed identical parameters ( $A_{413}/A_{280}$ ) as determined for apo-cyt  $b_5$  reconstituted with hemin chloride [11]. The overall yield of isolated cyt  $b_5$  protein relative to the protein amount in bacterial membranes was 13.3%. This value is lower than that obtained earlier (18.3%) [11] because of a shortened production time (from 4 to 1 h), however, 7.2 mg of cyt  $b_5$  protein was obtained from one liter of bacterial suspension.



**Figure 6.** SDS-PAGE of photo-cyt  $b_5$  samples during the purification (separated on 15% gel). Line **1** – homogenate from *E. coli* cells expressing cyt  $b_5$ ; line **2** – supernatant of the *E. coli* cells after sonication; line **3** – membrane fraction; line **4** – supernatant after solubilization; line **5** – final cyt  $b_5$  preparation after DEAE Sepharose CL-6B chromatography; **St** – refers to line with cyt  $b_5$  standard.

To confirm the applicability of photo-cyt  $b_5$  for mapping of protein-protein interactions among reaction partners of the MFO system, photo-cyt  $b_5$  was reconstituted in DLPC lipids with CYP2B4. After UV light photoactivation of diazirine group of photo-cyt  $b_5$ , three cross-links were detected on SDS-PAGE (see Fig. 7). The MALDI-TOF mass spectrometry analysis of their chymotryptic peptides revealed three covalent oligomers composed of CYP2B4 and cyt  $b_5$ . Based on their molecular weights, molar ratios of 1:1, 1:2, and 2:1 of CYP2B4 and cyt  $b_5$  in cross-links can be deduced. The formation of functional oligomeric complexes has been already demonstrated for various CYPs [18]. Moreover, a binary complex formed between CYP2B4 and cyt  $b_5$  has been successfully cross-linked by water soluble carbodiimide [19]. It is interesting to note that in our experiments the presence of diamantane, a high affinity binding substrate of CYP2B4 [20], markedly increased the amounts of oligomers formed (see Fig. 7). This observation is in line with the experimental data showing that the CYP substrate enhances the binding affinity of CYP for cyt  $b_5$  [5]. It seems that in the interaction between CYP and

cyt  $b_5$  several regions of these proteins are involved. The interaction by their cytosol-exposed regions has been predicted and experimentally proven [21]. However, our data suggest that CYP and cyt  $b_5$  interact also by their membrane anchors to form the reaction complex. Although the recent modeling describes orientations of CYP above and inserted in the membrane, no such precise data are available for cyt  $b_5$  [22]. To our best knowledge the protein-protein interaction between CYP and cyt  $b_5$  via their membrane anchors was directly proven for the first time. Application of the described approach will allow determining the mutual orientation of both proteins within the membrane environment and thus refining *in silico* models of CYP - cyt  $b_5$  interactions [23].



**Figure 6.** Photo-cross-linking of CYP2B4 and photo-cyt  $b_5$ . Photolysed reaction mixture containing CYP2B4 and photo-cyt  $b_5$  was separated on SDS-PAGE using 10% separation gel: line **1** – photo-cyt  $b_5$ , line **2** – CYP2B4 + photo-cyt  $b_5$ , line **3** – CYP2B4 + photo-cyt  $b_5$  + diamantane, line **4** – CYP2B4. Silver stained protein bands in violet frames are cross-links of CYP2B4 and cyt  $b_5$ .

## 5. CONCLUSION

The recombinant photo-cyt  $b_5$  was successfully prepared and proven to covalently link through its photo-reactive diazirine to CYP2B4 in the membrane environment. Thus, the photo-cyt  $b_5$  is applicable for mapping of protein-protein interactions among reaction partners of the MFO system.

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