1P106 無細胞タンパク質合成系を利用した新規膜タンパク質機能発現系の開発
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A Novel System for Functional Membrane Protein Production Using Cell-free Protein Synthesis into Liposome
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Cell-free expression has become a highly promising tool for the fast and efficient production of membrane proteins. We have investigated a novel cell-free system for the production of membrane proteins integrated into liposome. We used the light-driven proton pump, bacteriorhodopsin (BR) consisting seven transmembrane α -helices as a model membrane protein. To integrate a synthesized polypeptide into liposome, *in vitro* protein synthesis system based on *E.coli* extracts in the dialysis mode was supplemented various kinds of detergents and lipid. When the system was supplemented some detergents and lipid mixture, we could obtain functional BR in high amounts. According to analysis of the functional expression process, the protein synthesis was inhibited by the existence of detergent in the reaction solution. As the detergent removes by dialysis, protein synthesis and the formation of liposome initiate. Consequently, the protein synthesis coincides with the liposome formation. Thus, it is considered that lipid disks protect hydrophobic polypeptide and the proper folded membrane protein is efficiently produced into liposome. This approach might provide an efficient strategy for the production of functional membrane proteins at high level yielding for biochemical and biophysical studies. In this presentation, we will discuss the mechanism of functional synthesis of membrane proteins, and show also a possibility of this system as suitable tool for study of these.

1P108 高い代替オキシダーゼ活性を有するヤエヤマクワズイモミトコンドリア内膜の電子伝達タンパク質の解析
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Mitochondrial inner-membrane proteins of the spadix of *A. atropurpurea* with high AOX activity
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Spadices of Arales *Alocasia* is known to have high cyanide-resistant alternative oxidase (AOX) activity, one of the unique properties of plant mitochondrial respiratory chain. To analyze the protein composition and characterize the proteins participating in electron transfer, we prepared mitochondria from a male flower of *A. atropurpurea*. Using dodecylmaltoside (DM), the inner-membrane proteins were solubilized and separated by the DEAE chromatography. Elution with 0.1 M KCl eluent provided protein fraction 1 (F1) and that with 0.1-1.0 M KCl gradient did fraction 2 (F2). SDS-PAGE of F1 showed a specific band at 45 kDa, while F2 did two bands around 31 kDa, in addition to the common multiple bands between 50-66 kDa and below 14 kDa in both fractions. Optical absorption spectra of F1 exhibited absorption bands at 428, 454, and 478 nm, suggesting the inclusion of flavoprotein, whereas those of F2 did at 412, 450, and 478 nm, implying the contribution of heme protein. The fluorescence spectra of F1 and F2 were silent at pH 8.0, while at pH 3.0 it exhibited an emission band with the maximum at 530 nm, indicative of FAD. EPR of the both fraction also yielded a clear signal of flavo-semiquinone radical. Further, F2 fraction showed a typical spectrum of high-spin heme, together with those of low-spin heme and non-heme iron. Observation of the high-spin heme in mitochondrial fractions is a new finding since known cytochromes in complex III in animal cells have all low spin hemes and heme a₃ of complex IV are known to be EPR silent.

1P107 *Paracoccus denitrificans* チトクロム酸化酵素遺伝子の大腸菌無細胞転写翻訳系での発現に及ぼすYidC強制発現の効果
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Effect of overexpressed YidC on the expression of *P. denitrificans* cytochrome *c* oxidase gene in an *E. coli* cell-free system
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Cytochrome *c* oxidase (CcO) reduces dioxygen coupled to the proton pumping across the membrane. IR spectroscopic observation of changes in protonation state and polarity of amino acid functional groups during proton pumping is indispensable for elucidation of the proton pumping mechanism. Site-specific stable isotope labeling of an amino acid residue in a protein in a cell-free system is required for identification of the IR signals of the amino acid residues in the proton pumping system. We have succeeded in expressing *Paracoccus denitrificans* CcO genes in an *E. coli* cell-free system supplemented with heme A, Cu ion and *E. coli* membrane fractions at the yield of about 500 μ g/5 ml reaction mixture. However only about 1 % of the synthesized proteins are enzymatically active, while the others almost remained in aggregated state, implying that functional contribution of YidC which is proposed to mediate membrane insertion of quinol oxidase (PNAS 100, 5801-5806, (2003)), is insufficient. In this report, we enriched the membrane fractions with YidC. Enriched YidC with a C-terminal His-tag did not impair the protein synthesis. However, properly assembled fraction, estimated by the ferrocycytochrome *c* oxidation activity, did not increase significantly. These results suggest that instead of YidC, the Sec system, which is often indispensable for insertion of simple membrane proteins, facilitates the membrane insertion of the CcO subunits. Possibility of cooperation of YidC with the Sec system can not be excluded at present.

1P109 共鳴ラマン分光法によるウシ心筋ミトコンドリア呼吸鎖 NADH : ユビキノン酸化還元酵素の FMN と鉄硫黄クラスターの測定
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Resonance Raman spectra of the FMN and iron-sulfur clusters of the bovine heart NADH: coenzyme Q oxidoreductase.
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NADH: ubiquinone oxidoreductase (complex I) reduces ubiquinone by NADH at the entrance of the mitochondrial electron transfer chain. The electron transfer reaction is coupled to proton pumping. The enzyme is the largest component of the mitochondrial respiratory system with the proposed mass of 1000 kDa composed of 45 subunits. The putative redox-active sites include six EPR detectable iron-sulfur clusters, one FMN and several tightly bound ubiquinones.

In order to elucidate the reaction mechanism of complex I, spectral characterizations of these redox sites are prerequisite as well as X-ray structural analyses at high resolution. Although extensive EPR investigations for the iron-sulfur clusters have identified the redox and magnetochemical properties of the clusters, no spectral and functional study has been reported.

Resonance Raman technique is powerful for identification of a chromophore contained in a multichromophore system. However, contaminant proteins with much stronger absorptions than that of FMN and iron-sulfur cluster, like as cytochrome *c* oxidase, seriously obstruct accurate measurement of resonance Raman spectrum of complex I. The enzyme preparation purified from bovine heart mitochondria by our improved method contained a negligible amount of hemoproteins (less than 2% of cytochrome *c* oxidase (mole/mole)).

By using highly improved enzyme preparation and resonance Raman apparatus, the resonance Raman spectra of the FMN and the iron-sulfur clusters of complex I were determined upon excitation at 441.6nm.