1P.15 Knock-down of cytochrome *c* oxidase structural subunits in HEK293 cells

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Background: Mammalian cytochrome *c* oxidase (CcO), the terminal enzyme of respiratory chain, is a multiprotein complex of approximately 200 kDa and composed of 13 subunits. CcO assembly within the inner mitochondrial membrane is a sequential and relatively slow process. The aim of our study was to analyze an impact of downregulation of selected structural CcO subunits on CcO assembly. We chose Cox4 and Cox5a subunits, both of which enter the initial stage of the process and Cox6a subunit taking a part in a terminal step of CcO holocomplex assembly. **Methods:** The down-regulation of selected subunits was performed using RNA interference. We constructed 33 derivatives of pCMV-GIN-ZEO plasmid coding for hairpins targeted to different positions of *COX4I1, COX5A* and *COX6A1* mRNA, respectively. RNAi inducing transcript of pCMV-GIN-ZEO plasmid contains GFP and neomycin phosphotransferase coding sequences situated in tandem, upstream of the miR30-like hairpins. The recombinant plasmids were transfected to the HEK293 cells and the stable polyclonal cell populations were selected in a medium containing G418. **Results:** The depletion of Cox4, Cox5a and Cox6a was confirmed by SDS-PAGE immunoblot analyses. In selected cells with the lowest residual amounts of Cox4 and Cox6a subunits, transcripts of both tissue-specific isoforms were quantified by qRT-PCR. The depletion of Cox4, Cox5a and Cox6a subunits in HEK293 cells was accompanied by diminished amount of CcO holoenzyme and an altered assembly pattern. Lower content of CcO correlated with decreased CcO activity. Isolated CcO deficiency also manifested at the level of supercomplexes. The high-resolution respirometry showed normal normoxic maximally stimulated respiration after FCCP treatment (state 3u) in knockdown cells with Cox5a and Cox6a1 depletion but increased P50 values. **Conclusion:** Our results argue for importance of Cox4, Cox5a and Cox6a subunits for CcO biogenesis.

Supported by IGA MZ NS 10581/3 and MSM 0021620806.

doi:10.1016/j.bbabio.2010.04.063

1P.16 Structural and functional characterization of respiratory supercomplex containing complex III and IV from hyperthermophilic eubacterium *Aquifex aeolicus*

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Respiratory chain consists of several complexes named complex I to V according to their redox potentials during electron transfer. Complex I, III and IV couple the electron transfer to proton translocation and form a proton gradient across membrane which is used by complex V to produce ATP. The organization of these complexes has been studied for years and the term "supercomplex", representing several complexes in direct contact, was suggested. Here we report a supercomplex mainly containing fully assembled complex III and complex IV isolated from native membranes of *A. aeolicus*. Later a full complex IV and an incomplete complex III could be isolated from this supercomplex. All these three proteins were identified and characterized. The supercomplex mainly contains cytochrome *b* (*petB*), cytochrome c_1 (*cyc*), Rieske iron–sulfur protein (*petA*), cytochrome *c* oxidase subunit I (*coxA2*) and subunit II (*coxB2*). Isolated complex IV consists of subunit I and II. The incomplete complex III lost Rieske iron–sulfur protein during isolation. EPR spectra of the supercomplex showed signals from low-spin cytochrome b_H, b_L and cytochrome c_1 as well as signals of an intensive copper and a high-spin heme. Copper and high-spin heme signals were also detected in the complex IV. Metal content measurement indicates four coppers in each supercomplex and complex IV. The supercomplex catalyzes cytochrome *c* oxidation and reduction, quinol oxidation and oxygen consumption; complex IV catalyzes cytochrome *c* oxidation, quinol oxidation and oxygen consumption; and the incomplete complex III is able to catalyze cytochrome *c* reduction without Rieske iron–sulfur protein. Stoichiometry of the supercomplex was analyzed by LILBID (Laser Induced Liquid Bead Ion Desorption), which indicates two molecules of complex III and one molecule of complex IV in each supercomplex. To summarize all these results, possible models and electron transfer schemes of this supercomplex are proposed.

doi:10.1016/j.bbabio.2010.04.064

1P.17 Localizing the ubiquinone binding site of the *Escherichia coli* complex I

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The NADH:ubiquinone oxidoreductase, the respiratory complex I, is the entrance point of electrons into the respiratory chain. Complex I transfers two electrons from NADH to ubiquinone and couples this reaction with the transport of four protons across the membrane. The position and structure of the ubiquinone binding site within the complex is not known. We generated several variants of the enzyme by *Lambda*-Red Recombineering bearing cysteine residues at suitable positions on the surface of the protein. To these cysteine residues (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl)-methanethiosulfonate (MTSL), a spin-probe, was attached. In addition, a derivative of pentyl- and decyl-ubiquinone was synthesized containing the MTSL label at the distal position of the alkyl-chain. In experiments with the spin-probe bound to decyl-ubiquinone and bound to the protein, distances between the spin-probes were measured pairwise by pulsed EPR spectroscopy (ELDOR). The positions of the cysteine residues were chosen that they allow localizing the quinone binding site via triangulation. First results have been obtained in cw-experiments using the NuoB R112C variant in combination with the two different ubiquinone derivatives demonstrating that the method is suitable for measuring distances within the protein and between distinct protein sites and the quinone binding site.

doi:10.1016/j.bbabio.2010.04.065

1P.18 Sodium interaction of the complex I antiporter-like subunits NuoL, M and N from *Escherichia coli* studied by ^{23}Na NMR

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Complex I (NADH:quinone oxidoreductase) proteins NuoL, NuoM and NuoN are homologous to one type of Na^+/H^+ antiporters. Thus, these subunits are prime candidates for harbouring important parts of the proton pumping machinery. If they also retain antiporter function and/or the ability to pump sodium has been a matter of debate. The aim of this study was to investigate the sodium binding properties of the NuoL, NuoM and NuoN subunits from *E. coli* complex I and compare them to those of the *bona fide* antiporters MrpA and MrpD from *Bacillus subtilis* using ^{23}Na NMR spectroscopy. This technique has been demonstrated to be particularly suitable to monitor ion binding properties of macromolecules under conditions of very fast chemical exchange [1, 2], as expected from a transporter protein. High amounts of the individual proteins were obtained by expressing them as cytochrome c fusion proteins in *E. coli*. Purification was facilitated by a C-terminal histidine tag fused to the cytochrome c domain. The mobility of Na^+ in the presence of the antiporter proteins was measured at gradually increasing concentrations. Subsequently, the concentration of another, non-interacting cation, NH_4^+, was increased, while sodium was kept constant. Cytochrome c alone was used as negative control protein. From this data, specific binding constants for Na^+ could be estimated for each of the five proteins. The Na^+ interaction was then assessed under different conditions and pH, in the presence of quinone and in the presence of the sodium-hydrogen exchange inhibitor 5-ethylisopropyl amiloride (EIPA). The sodium interaction was compared to the real antiporters MrpA and MrpD.

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doi:10.1016/j.bbabio.2010.04.066

1P.19 Infrared spectroscopic analysis on the substrate induced conformational flexibility of the NADH:ubiquinone oxidoreductase

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Protein dynamics play an important role in the catalytic efficiency of enzymes and conformational changes may take place during the substrate binding of the NADH:ubiquinone reductase, the respiratory complex I. A coupled FTIR spectroscopic and perfusion induced approach was applied that provides the possibility recording $^1H/^2H$ exchange kinetics at the level of the amide proton in the mid infrared (170–1500 cm^{-1}). This approach is extremely sensitive to tertiary structure changes [1–3]. In general the exchange rates depend on hydrogen bonding and solvent accessibility. It was suggested that protein structure can be divided in three types of structure characterized by their particular ($^1H/^2H$) exchange dynamics. These three domain types are clearly distinguishable for complex I and the soluble NADH binding fragment and their relative ratios depend on the presence of bound substrate. Furthermore the spectral signature of the overall internal hydrogen bonding was probed in the far infrared (300 to 30 cm^{-1}). The interest of this spectral range is based on the observation that the far-infrared contribution of a wide range of molecules is dominated by vibrations involving a substantial fraction of the atoms forming the molecule and motion associated with intermolecular hydrogen bond vibrations [4,5]. Due to their collective nature, such modes are highly sensitive to the intra- and intermolecular structure and thus provide a unique fingerprint of the conformational state of the molecule and effects of its environment. We use these two infrared spectroscopic approaches to learn about the conformational flexibility of the respiratory NADH:ubiquinone oxidoreductase (complex I) induced by various substrates and present evidence for a different effect of NADH and of NADPH. The role of the quinone for the conformational flexibility is discussed.

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doi:10.1016/j.bbabio.2010.04.067

1P.20 Tempering of the cytochrome bc_1 complex of *Rhodobacter capsulatus* by pH

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The cytochrome bc_1 complex is a dimeric membrane ubiquinol: cytochrome *c* oxidoreductase. After an ubiquinol molecule is oxidized in the catalytic center *P* close to the positively charged side of the coupling membrane, the two released electrons going to different acceptors. One is taken by the mobile domain of the [2Fe–2S] iron–sulphur Rieske protein to be passed further to the *c*-type cytochromes. The other electron crosses the membrane, via the low- and high-potential hemes of cytochrome *b*, to reduce a stable semiquinone molecule which is steadily maintained in one of the two centers *N* from the opposite membrane side [1]. The kinetics of flash-induced generation of membrane voltage by the cytochrome bc_1 complex can be traced via spectral shifts of native carotenoid pigments and correlated with the kinetics of electron transfer as measured in the same samples. Earlier we have shown, at neutral pH values, that small amounts of Zn^{2+} ions could make the flash-induced redox-reactions of cytochrome *b* visible, apparently, by retarding the oxidation of heme b_h. Binding of a Zn^{2+} close to the center *P* not only retarded the proton release from this center and the movement of the FeS domain towards cytochrome c_1, but also slowed down the oxidation of heme b_h and the formation of ubiquinol in center *N*. This correlation was attributed to the earlier postulated mechanistic coupling between the two quinone-binding centers [2]. In the case of such coupling, however, one could expect that the retardation of events in center *N* should, reciprocally, affect the events in center *P*. Here we show that the same kinetic behaviour of the cytochrome bc_1 complex could be observed at high pH, when the protonation of ubiquinol in center *N* is retarded. These observations support our suggestion of a cross-