S4.17 The activity of exogenous NAD(P)H dehydrogenases in *Acanthamoeba castellanii* mitochondria

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The mitochondrial respiratory chain of plants and fungi contains multiple NAD(P)H dehydrogenases. In addition to Complex I there are two rotenone-insensitive dehydrogenases located on the outer surface of the inner mitochondrial membrane (ND_{ex}), i.e., external NADH and external NADPH dehydrogenases. Using bioenergetic methods (oxygen consumption and membrane potential measurements) we have investigated protozoan *A. castellanii* mitochondria in order to study the activity of ND_{ex}. We have determined the activity of both NADH and NADPH dehydrogenases with the maximum value at pH 6.8, likewise the cyanide-resistant alternative oxidase activity. It seems to be consistent with the putative role of these enzymes which probably cooperate with each other and very likely constitute a wasteful system preventing overreduction of the electron transport components in the respiratory chain. We have also examined a Ca^{+2}-dependence of *A. castellanii* ND_{ex} activities. Our data show that NADH dehydrogenase is probably slightly or not sensitive to Ca^{+2} ions in contrast to NADPH dehydrogenase, which is Ca^{+2}-sensitive and this sensitivity increases with the rise of pH value.

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S4.18 NADH induced conformational changes in *E. coli* complex I measured by site-directed spin-labelling

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We have probed the extent of local structural changes of the *E. coli* complex I upon the addition of nucleotides by site-directed spin-labelling. Surface cysteine variants of complex I have been generated, prepared and spin-labelled with a sulfhydryl-specific nitroxide reagent. The NADH:decyl-ubiquinone activity of the labelled enzyme variants was unchanged compared to the wild type enzyme, indicating the negligible influence of the label on the physiological activity. From the line shape of the spectra, the spin-label mobility was deduced, which is a direct measure of the local protein environment. All measured mobilities of the nitroxide side chains reflected the fold of the protein as deduced from the crystal structure and secondary structure prediction. For sites where a restricted mobility of the label indicated contacts with neighbouring amino acids, a change in the labels EPR-spectrum was observed upon the addition of NADH, whereas the addition of NAD^+ had no effect. Therefore we were able to pinpoint the observed conformational changes of the *E. coli* complex I to distinct secondary structure elements within individual subunits.

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S4.19 Assembly of the *Escherichia coli* NADH:ubiquinone oxidoreductase (complex I)

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The bacterial complex I generally consists of 14 different subunits. In contrast to the mitochondrial complex, where several distinct assembly intermediates were detected, the assembly of the bacterial complex was not examined until now. We used *Escherichia coli* strains in which the *nuo*-genes coding for the subunits of complex I are individually disrupted by insertion of a resistance cartridge and studied the assembly of the complex in the mutants. The cytoplasmic membranes of these mutants exhibited no complex I specific activity. However, in the cytoplasm of some mutants the subunits and cofactors of the soluble NADH dehydrogenase fragment were detected. The mutants lacking either *nuoK* or *nuoL* contain a larger membrane-bound complex I fragment, which exhibits NADH/ferricyanid but no physiological activity. The fragment from the *nuoL* mutant strain was purified and characterised. The role of NuoL in the mechanism of complex I is elucidated by the activities of this partially assembled complex I.

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S4.20 Substrate crosstalk involving conformational changes in *E. coli* respiratory complex I

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The respiratory complex I couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane. An addition of NADH but not NADPH to the *E. coli* complex I led to conformational changes identified by electron microscopy and CD-spectroscopy. EPR spectroscopy revealed that both, NADH and NADPH, completely reduce the Fe/S-clusters of the enzyme. However, the enzymatic activity with NADPH is, in contrast to the activity of NADH, not coupled with proton translocation and not sensitive to inhibitors of the quinone site. Instead, the reaction with NADPH led to an increased production of superoxide radicals. We propose, that the binding of NADH and not the reduction of the enzyme leads to conformational changes needed for opening the ubiquinone binding site.

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S4.21 Monitoring the conformational change of the NADH dehydrogenase fragment from the *E. coli* complex I by attenuated total reflexion spectroscopy

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The proton-pumping NADH:ubiquinone oxidoreductase (complex I), the first complex of the respiratory chains, couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane. The soluble NADH dehydrogenase fragment represents the electron input part of complex I and comprises 3 subunits, one flavin mononucleotide and up to six iron–sulfur clusters. Previous electron microscopic studies revealed NADH induced large conformational changes for complex I. Structural information on the soluble fragment about these changes are not available. ATR-FTIR spectroscopic investigations of the conformational change of the NADH dehydrogenase fragment in the presence and absence of NADH substrate have been applied. The secondary structure determination was based on the analysis of the intense amide I band between 1710 to 1600 cm^{-1} which predominantly includes the C=O stretching vibration of the protein backbone. The highly organized α helical and β sheet components are negligibly altered upon NADH addition whereas variations for turns and random elements are obtained. During perfusion-induced investigations an increase of the amide I and amide II are observed which indicates a conformational change upon NADH binding. Furthermore, investigations on the whole complex I are presented.

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S4.22 RP-HPLC AND ESI/MS/MS subunit analysis of bovine heart complex I: Application for identifying ROS-induced modifications

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An effective method was developed for isolation and analysis of bovine heart Complex I subunits. The method utilizes C18 reversed-phase HPLC and a water:acetonitrile gradient containing 0.1% trifluoroacetic acid. Employing this system, 36 of the 45 Complex I subunits eluted in 28 distinct chromatographic peaks. Nine subunits did not elute including B14.7, MLRQ and the seven mitochondrial-encoded subunits. The method, with UV detection, is suitable for either analytical (<50 μg protein) or preparative (>250 μg protein) applications. Subunit(s) eluting in each chromatographic peak were initially determined by MALDI-TOF/MS with subsequent positive identification by reversed-phase HPLC-ESI/MS/MS analysis of tryptic digests. In the latter case, subunits were identified with a 99% probability using Scaffold analysis software combined with a X! Tandem protein search engine. The RP-HPLC subunit analysis method represents a major improvement over previous separation methods with respect to resolution, simplicity and ease of application. As expected, the HPLC method, combined with mass spectrometry analysis is capable of detecting oxidatively damaged subunits after *in vitro* exposure of Complex I to peroxynitrite. The results of these experiments will help elucidate the chemical nature of oxidative damage to this key electron transport chain component. Supported by NIH GMS (24795) and Welch Foundation (AQ1481).

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S4.23 Heterologous expression, isolation and characterization of A subcomplex of the respiratory complex I from *Aquifex aeolicus*

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The proton-pumping NADH:ubiquinone oxidoreductase (complex I) is the first complex of the respiratory chains in many bacteria and most eukaryotes. It is the least understood respiratory complex, due to its size and intricate assembly. Complex I of the hyperthermophilic bacterium *Aquifex aeolicus* is made up of 13 different subunits named NuoA2–N1. The flavoprotein subcomplex contains subunits NuoE and F and is considered as the simplest catalytically active NADH dehydrogenase. It harbours one FMN, the binuclear iron–sulfur cluster N1a and the tetranuclear cluster N3 as well as the NADH binding site. This subcomplex was heterologously overproduced in *Escherichia coli* and isolated by standard chromatographic techniques. The proteins of the preparation were identified as NuoE and NuoF by N-terminal sequencing and mass spectrometry. The expected cofactors of the preparation were detected by EPR- and UV/vis spectroscopy. The preparation is thermostable and catalyzes NADH/ferricyanide oxidoreductase activity at temperatures up to 85 °C. Furthermore, the preparation is homogenous as shown by dynamic light scattering and meets all prerequisites for crystallization attempts.

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S4.24 Modelling, cloning and expression of the *P. falciparum* NADH dehydrogenase

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The respiratory chain of the human malaria parasite *Plasmodium falciparum* lacks a canonical protonmotive NADH:ubiquinone oxidoreductase (Complex I), containing instead a single-subunit, non-protonmotive Ndh2, similar to that found in plant mitochondria, fungi and some bacteria. As such, the *P. falciparum* Ndh2 (PfNdh2) presents itself as an attractive anti-malarial chemotherapeutic target, and we have developed a structural model and heterologous expression system for this enzyme to facilitate its physicochemical and enzymological characterisation. Structural modelling of PfNdh2 suggests the presence of two Rossman folds forming the flavin and NADH binding sites, with membrane attachment via a C-terminal amphipathic helix. Heterologous expression of PfNdh2 in *E.coli* NADH dehydrogenase knockout strain ANN0222 yields an active enzyme with Km values for NADH and decylubiquinone of 14- and 12 μM respectively in crude membrane preparations. In addition, the recombinant PfNdh2 is strongly inhibited by 1-hydroxy-2-dodecyl-4(1H)quinolone (IC50=70 nM in crude membrane preparations) with decylubiquinone as substrate. Purification of His-tagged PfNdh2 for high throughput drug screening, stopped-flow spectrophotometry and NMR/FTIR spectroscopic investigation is currently underway.

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