1P.1 Structural insights into complex I obtained from new subcomplex Iδ

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Mitochondrial NADH:ubiquinone oxidoreductase (complex I) is an L-shaped membrane protein that contains at least 40 different subunits. Until a high-resolution structure of the holo-complex becomes available, the analysis of subcomplexes can provide useful information about the overall structural organisation of complex I. Exposing complex I purified from *Yarrowia lipolytica* to the chaotropic detergent N,N-dimethyldodecylamine N-oxide (LDAO) resulted in formation of a new 820 kDa subcomplex, termed Iδ. As determined by combining dSDS-electrophoresis, LILBID- and ESI-mass spectrometry, subcomplex Iδ harbours 31 subunits and comprises the hydrophilic subcomplex Iλ and the membrane-bound subcomplex Iβ. The missing subunits were ND1, ND2, ND3, ND4L, NUPM, NUXM, NB6M, NIMM and ST1. Subcomplex Iδ showed full non-physiological NADH:HAR activity and contained all EPR detectable Fe–S clusters. However, using the ubiquinone analogs DBQ or Q_1 as substrates no inhibitor-sensitive catalytic activity was detectable and could also not be recovered by addition of lipids. This indicated that subcomplex Iδ had lost its native enzymatic function. Structural characterisation of subcomplex Iδ by single particle electron microscopy revealed a structure in which the peripheral arm and a large fragment of the membrane arm appeared to be tethered by a thin connection. These findings suggest that in subcomplex Iδ the boot-shaped parental complex I had lost its "heel", i.e. a part roughly corresponding to subcomplex Iγ. Implications for the arrangement and functional roles of subcomplexes Iα, Iβ, Iλ and Iγ and the position of subunit ND6 in subcomplex Iδ will be discussed.

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1P.2 External NAD(P)H dehydrogenases in amoeba *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 at least two rotenone-insensitive dehydrogenases located on the outer surface of the inner mitochondrial membrane (ND_{ex}), i.e. external NADH and external NADPH dehydrogenases. We have investigated protozoan *A. castellanii* mitochondria in order to find the activity of ND_{ex}. We have determined the activity of both external 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 likely constitute a wasteful system preventing overreduction of the electron transport components in the respiratory chain. NADH dehydrogenase is probably slightly or not sensitive to Ca ions in contrast to NADPH dehydrogenase, which is Ca-sensitive. Under enzyme optimal conditions, *A. castellanii* mitochondria reveal a higher substrate affinity for external NADPH than for external NADH. Using blue-native polyacrylamide gel electrophoresis and histochemical staining (NBT + substrate-NADH and NADPH, respectively) we have identified ND_{ex} activities in solubilized *A. castellanii* mitochondria.

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1P.3 Characterisation and crystallisation of intact complex I from *Thermus thermophilus*

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NADH–ubiquinone oxidoreductase (complex I) is the first and the largest enzyme in the respiratory chain of mitochondria and most bacteria. Complex I catalyses the transfer of two electrons from NADH to quinone, coupled to the translocation of about four protons across the membrane. The mitochondrial enzyme contains 45 different subunits, while the bacterial enzyme consists of 13–15 different subunits. Analogues of all conserved bacterial subunits are found in the mitochondrial enzyme. Previously, we have determined the crystal structure of the hydrophilic domain of complex I from *Thermus thermophilus*. However, the high-resolution structures of the hydrophobic domain or the intact complex, as well as the coupling mechanism, remain unknown. The *T. thermophilus* complex I is studied here as a minimal model of the mitochondrial enzyme. A procedure for purification of intact complex I from *T. thermophilus* was developed. All subunits of the enzyme have been identified by peptide mass fingerprinting. Single-particle EM analysis showed that the *T. thermophilus* complex I has the typical L-shape. It shows high specific activity of electron transfer from NADH to decylubiquinone, which is sensitive to inhibitors rotenone and piericidin-A. Extensive crystallisation trials identified several different crystal forms, all containing intact complex I. The preliminary findings from crystallographic data will be discussed.

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1P.4 Electron pathways in mitochondrial complex I

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Complex I plays a central role in cellular energy production, transferring two electrons from NADH through a series of iron–sulfur clusters (FeS) to ubiquinone (CoQ); the electron transfer is coupled to the translocation of protons across the membrane. The FeS center N2 is the last acceptor in the electron-transfer chain, but the mechanism through which the enzyme couples the $1e^-$ reduction of the FeS centers to the $2e^-$ reduction of ubiquinone ($Q \rightarrow SQ \rightarrow QH2$) is unclear [1]. We identify two different families of inhibitors of complex I activity (class A and B) acting on the electron-transfer with different mechanisms. EPR data are coupled to fluorescence measurements on the effect of inhibitors on reactive

oxygen species (ROS) production and to enzymatic activity assays. Submitochondrial particles (SMP) were treated with class A or B inhibitors. NADH addition initiated the electron transfer. In our system class A inhibitors (rotenone, piericidin A) increase ROS production from complex I, whereas class B inhibitors (stigmatellin, mucidin, CoQ2) have no effect on ROS production. We measured the presence of semiquinone (SQ) at 180 K and state of reduction of the iron sulfur cluster N2 at 12 K in SMP inhibited with class A and class B inhibitors. Our data confirm a strong SQ signal reduction in the presence of rotenone while the signal intensity is less reduced in samples treated with stigmatellin [2]. N2 spectra show different reduction state in presence of rotenone and stigmatellin. In presence of stigmatellin the center is mainly oxidized. We hypothesize a two-step reduction performed by N2, possibly following a rearrangement of the site [3]. Rotenone like inhibitors, not allowing the access of the quinone to the active site, would block the enzyme in a conformation that only permits electron delivery from N2 to oxygen. In this conformation hydrophilic quinones like CoQ1 can be reduced by N2 to a semiquinone species in a non physiological site. This semiquinone can rapidly react with molecular oxygen to form anion superoxide. Stigmatellin like inhibitors would block the enzyme in a conformation allowing only the first step of quinone reduction (Q→SQ) in the physiological reduction site, but blocking any further reduction; this conformation does not allow reaction of N2 with oxygen.

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1P.5 NADH binding to complex I: Implications for the mechanism

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Complex I plays a central role in cellular energy production, coupling electron transfer between NADH and quinone to proton translocation. We have determined several X-ray structures of the oxidized and reduced hydrophilic domain of complex I from *Thermus thermophilus* at up to 3.1 Å resolution. The structures reveal the mode of interaction of complex I with NADH, explaining known kinetic data and providing implications for the mechanism of ROS production at the flavin site of complex I. Bound metals were identified in the channel at the interface with the frataxin-like subunit Nqo15, indicating possible iron-binding sites. Conformational changes upon reduction of the complex involve adjustments in the nucleotide binding pocket, as well as small, but significant, shifts of several α-helices at the interface with the membrane domain. These shifts are likely to be driven by the reduction of nearby Fe–S clusters N2 (the electron donor to quinone) and N6a/b. Cluster N2 is coordinated by unique motif involving two consecutive (tandem) cysteines. An unprecedented "on/off switch" (disconnection) of coordinating bonds between the tandem cysteines and this cluster was observed upon reduction. Comparison of the structures suggests a novel mechanism of coupling between electron transfer and proton translocation, combining conformational changes and protonation/de-protonation of tandem cysteines.

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1P.6 The role of the isolated [2Fe–2S] cluster adjacent to the flavin mononucleotide of mitochondrial complex I: Does it influence catalysis at the flavin site?

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Complex I is the proton pumping NADH:ubiquinone oxidoreductase of the mitochondrial inner membrane. Complex I from bovine mitochondria contains eight iron–sulphur clusters (two [2Fe–2S] clusters and six [4Fe–4S] clusters). Seven of them link the NADH oxidation site to the ubiquinone reduction site. The eighth cluster, named 2Fe[24] or N1a, is ligated by the 24 kDa subunit; it is isolated from the main chain of clusters but adjacent to the flavin mononucleotide and close enough to accept electrons from it. Whether the 2Fe[24] cluster has a role in the mechanism of complex I is not known. It is possible it minimises the lifetime of the semi-reduced flavin species, decreasing the rate of superoxide production and/or preventing direct hydrogen peroxide production by the fully reduced flavin. Complex I from *Escherichia coli* contains a homologous cluster with a reduction potential 0.1 V higher than that of the bovine cluster; complex I from *E. coli* also produces hydrogen peroxide rather than superoxide. The *E. coli* cluster is probably reduced during catalytic turnover, and so may be incapable of minimising the semi-reduced flavin. In this study, complex I from *Yarrowia lipolytica* was used to establish the role of the 2Fe[24] cluster. Mutations were generated in the closely homologous NUMH subunit, to increase the reduction potential of the [2Fe–2S] cluster to that observed in *E. coli*. The effects on catalysis and superoxide production by the complex are described.

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1P.7 Mitochondrial acyl carrier proteins in *Yarrowia lipolytica*: Guilty by affiliation with complex I

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Mitochondrial acyl carrier proteins (ACPMs) were first discovered in the 1980s in *Neurospora crassa*. They are thought to be involved in mitochondrial fatty acid synthesis and in the production of octanoic acid via a phosphopantetheine group covalently attached to a conserved serine. Our group has previously demonstrated that *Yarrowia lipolytica* codes for two different mitochondrial acyl carrier proteins, ACPM1 and ACPM2, that both are *bona fide* subunits of complex I. Deletion of the ACPM1 gene is lethal, whereas ACPM2Δ strains are viable in a certain strain background. However, the ACPM2Δ cells showed an apparent lack of complex I, pointing towards a role in assembly/stability for the complex. In contrast, ACPM1 seems to have a function beyond complex I. The two ACPM protein sequences differ mostly in their putative mitochondrial targeting sequences. We thus created a protein consisting of the ACPM1 targeting sequence fused to the sequence of mature ACPM2. Two DNA constructs with different length of the putative ACPM1 targeting sequence were created and used for plasmid-based complementation of the ACPM1Δ strain. No viable spores were obtained, indicating that both chimeric proteins failed to take over the function of ACPM1. In the ACPM2Δ strain, both constructs led to the formation of assembled complex I, suggesting that the functional difference between the ACPM variants is mediated by the targeting sequence. Currently, various domain-swap constructs are underway