Structure–function relationships in mitochondrial complex I of the strictly aerobic yeast *Yarrowia lipolytica*

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Abstract

The obligate aerobic yeast *Yarrowia lipolytica* has been established as a powerful model system for the analysis of mitochondrial complex I. Using a combination of genomic and proteomic approaches, a total of 37 subunits was identified. Several of the accessory subunits are predicted to be STMD (single transmembrane domain) proteins. Site-directed mutagenesis of *Y. lipolytica* complex I has provided strong evidence that a significant part of the ubiquinone reducing catalytic core resides in the 49 kDa and PSST subunits and can be modelled using X-ray structures of distantly related enzymes, i.e. water-soluble [NiFe] hydrogenases from *Desulfovibrio* spp. Iron–sulphur cluster N2, which is related to the hydrogenase proximal cluster, is directly involved in quinone reduction. Mutagenesis of His^{226} and Arg^{141} of the 49 kDa subunit provided detailed insight into the structure–function relationships around cluster N2. Overall, our findings suggest that proton pumping by complex I employs long-range conformational interactions and ubiquinone intermediates play a critical role in this mechanism.

Complex I from *Yarrowia lipolytica*

Proton pumping NADH:ubiquinone oxidoreductase (complex I) is the last unvanquished summit in the mitochondrial respiratory chain. Bovine heart complex I that has been well characterized by EPR spectroscopic and proteomic approaches [1,2], consists of 46 subunits, seven of which are encoded in the mitochondrial genome. It contains one molecule of non-covalently bound FMN and eight iron–sulphur clusters as redox-prosthetic groups. Although it is well known that electron transfer from NADH to ubiquinone is linked to the translocation of four protons across the inner mitochondrial membrane [3,4], the reaction mechanism of complex I is still unknown and has been the subject of a lively debate in recent years [5,6]. No high-resolution structure is available for complex I. Low-resolution structures obtained by electron microscopic single particle analysis show an L-shaped structure, consisting of a hydrophilic peripheral and a hydrophobic membrane arm, orientated perpendicular to each other [7–11].

With the aim to apply the powers of yeast genetics to complex I research, we have established the obligate aerobic yeast *Y. lipolytica* as a new model system [12]. Besides the respiratory chain complexes present in mammalian mitochondria, this organism also possesses an alternative NADH dehydrogenase (NDH2) and an alternative terminal oxidase. The latter two enzymes that are typically found in plants and fungi consist of single subunits and are unable to pump protons. Since the active site of NDH2 as found in standard laboratory strains of *Y. lipolytica* is orientated towards the external face of the mitochondrial inner membrane, this enzyme cannot complement complex I function. Only when NDH2 was artificially redirected to the inner face of the mitochondrial inner membrane [13], deletions of the genes for essential subunits of complex I were achieved, which then could be complemented with site-directed mutant versions borne on replicative plasmids [12].

Addition of a His_6 tag to the C-terminus of the 30 kDa subunit permitted fast and efficient affinity purification of *Y. lipolytica* complex I [14]. Using a combination of proteomic and genomic analyses, a total of 37 subunits could be identified. dSDS/PAGE (where dSDS stands for doubled SDS) [15], in combination with MALDI–TOF MS (matrix-assisted laser-desorption ionization–time-of-flight mass spectroscopy) of tryptic fragments and N-terminal sequencing has allowed the identification of 26 proteins as *bona fide* subunits of *Y. lipolytica* complex I [16]. From the 14 central subunits, which are homologous with the constituents of prokaryotic complex I [17], the seven nuclear-coded proteins and two of the seven mitochondrially coded proteins could be identified. The remaining five mitochondrially coded central subunits could be tentatively assigned from their positions above the electrophoretic diagonal in dSDS gels and their predicted molecular masses.

With few exceptions, the function of most of the 23 so-called accessory subunits of *Y. lipolytica* complex I is unknown. Strikingly, a significant number of them are STMD

Key words: aerobic yeast, cluster N2, mitochondrial complex I, NADH:ubiquinone oxidoreductase (complex I), proton pump, *Yarrowia lipolytica*.

Abbreviations used: NDH2, alternative NADH dehydrogenase; STMD, single transmembrane domain.

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Table 1 | STMD subunits of complex I from mammals, fungi and yeasts*

Mol. mass, molecular mass; N.I., not identified.

SwissProt nomenclature	Name	*Bos taurus* (14/46 subunits)		*N. crassa* (6/38 subunits)		*Y. lipolytica*† (6/37 subunits)	
		Mol. mass (kDa)	Accession no.	Mol. mass (kDa)	Accession no.	Mol. mass (kDa)	Accession no.
NIMM	MWFE	7.5	Q02377	9.8	CAE85571	(9.8)	Genome hit
NB2M	B12	12	Q02365	10.6‡	XP_331394	(9.4)	Genome hit
NESM	ESSS	14.5	Q8HXG5	11.7‡	XP_324110	23.4§	YALI0E29095g
NB5M	B15	15	P48305	7‡	XP_322246	10.4‖	Genome hit
NB6M	B16.6	16.6	Q95KV7	13.5‡	EAA29209	(14.1)	Genome hit
NIAM	ASHI	19	S28242	20.1‡	XP_332152	14.6	YALI0D04939g
NIKM	KFYI	6	Q02376	N.I.		N.I.	
NINM	MNLL	7	Q02378	N.I.		N.I.	
NIGM	AGGG	8	Q02374	N.I.		N.I.	
NI9M	B9	9	Q02371	N.I.		N.I.	
NUML	MLRQ	9	Q01321	N.I.		N.I.	
N4AM	B14.5a	14.5	Q05752	N.I.		N.I.	
NISM	SGDH	16	Q02380	N.I.		N.I.	
NB7M	B17	17	Q02367	N.I.		N.I.	

*Based on the work of Abdrakhmanova et al. [16] and Cardol et al. [39].

†In cases where the N-terminus has not been determined, molecular masses of precursors are given in brackets. The annotated *Y. lipolytica* genomic sequence can be found at http://cbi.labri.fr/Genolevures/.

‡Molecular masses predicted from sequence analyses only. (If not indicated otherwise, molecular masses of nuclear coded subunits are based on data from proteomic analyses.)

§Formerly called NUWM.

‖Formerly called NUVM.

(single transmembrane domain) proteins with small, charged extramembranous domains (Table 1). Six STMDs are found in fungi and 14 in mammalian complex I. It is tempting to speculate that the common functional requirements for this class of subunits are structure rather than sequence specific, since the total number of STMD subunits differs between phyla, and sequence conservation is generally low among orthologous groups [16]. They may function in a chaperone-like manner during assembly of the membrane arm. A representative of this class, the NUWM subunit of *Y. lipolytica*, which was only recently found to represent the homologue of the bovine ESSS subunit, was shown to be present in a hydrophobic fragment after LDAO cleavage and localized in the membrane arm using electron microscopic single particle analysis [16].

A homology model for the 49 kDa and PSST subunits of complex I

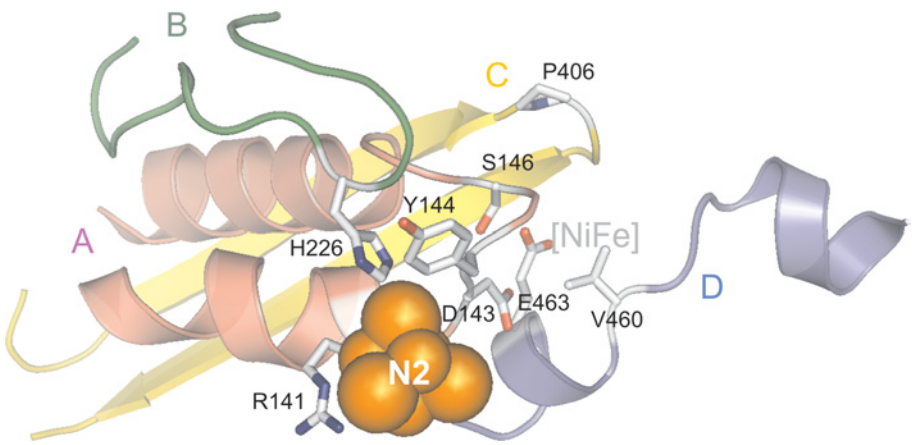
Several subunits of complex I are distantly related to water-soluble and membrane-bound [NiFe] hydrogenases from various eubacterial and archaebacterial sources. X-ray structures of water-soluble [NiFe] hydrogenases from *Desulfovibrio* spp. that are made up of only two subunits are available [18–20] and have been used to construct models for the homologous 49 kDa and PSST subunits of complex I.

Sequence alignments between the two subunits of water-soluble [NiFe] hydrogenases, their homologues from membrane-bound [NiFe] hydrogenases and complex I sequences from various organisms, in combination with secondary structure predictions [21], have revealed that major structural elements from the large and small subunits of water-soluble [NiFe] hydrogenases seem to be conserved in the 49 kDa and PSST subunits of complex I. Prominent examples are two large β-sheets, close to the N- and C-terminus of the 49 kDa subunit, each composed of three antiparallel β-strands and a three or four stranded parallel β-sheet in the PSST subunit.

Even more strikingly, the structural elements of the *Desulfovibrio fructosovorans* large subunit [20] that make close contact to the [NiFe] active site are conserved in the 49 kDa subunit of complex I [22,23]. Four elements in the hydrogenase structure contribute to this conserved fold (Figure 1): element A is the helical domain around the two N-terminal ligands of the [NiFe] site (Cys^{72} and Cys^{75}, corresponding to Asp^{143} and Ser^{146} in *Y. lipolytica*), element B is the loop containing His^{228} (corresponding to His^{226} in *Y. lipolytica*), element C is formed by the two β-strands around Pro^{475} (corresponding to Pro^{406} in *Y. lipolytica*), and element D is the domain around the two C-terminal ligands of the [NiFe] site (Cys^{543} and Cys^{546}, corresponding to Val^{460} and Glu^{463} in *Y. lipolytica*).

Figure 1 | Homology model of structural elements in the 49 kDa subunit of complex I that participate in forming the ubiquinone reducing catalytic core

Structural elements A to D taken from the structure of *D. fructosovorans* [NiFe] hydrogenase large subunit (Protein Data Bank number 1FRF) are shown in colour. Residues from the 49 kDa subunit of *Y. lipolytica* that correspond to key residues within these structural elements were placed into the hydrogenase structure. These side chains are coloured according to CPK (Corey–Pauling–Koltun) conventions and labelled according to their positions in the *Y. lipolytica* subunit precursor. Iron–sulphur cluster N2, corresponding to the proximal iron–sulphur cluster in the hydrogenase small subunit is also shown. See text for further details.



Site-directed mutagenesis of the 49 kDa and PSST subunits of complex I

Site-directed mutagenesis of the 49 kDa and PSST subunits of *Y. lipolytica* complex I resulted in three types of effects: (i) alterations of the EPR signature of cluster N2, (ii) effects on binding of quinone substrates or quinone-like inhibitors and (iii) loss of catalytic activity. When the effects of mutations in the 49 kDa and PSST subunits of *Y. lipolytica* complex I [22,24–27] and other model organisms like *Rhodobacter capsulatus* [28], *Neurospora crassa* [29], *Escherichia coli* [30] and *Helicobacter pylori* [31], are compared with their map positions in the hydrogenase model, a striking picture emerges (Figure 2). Mutations of the cysteine ligands of cluster N2 in the PSST subunit, generated in *N. crassa* or *E. coli* lead to inactivity and the absence of the cluster N2 EPR signals. Strong interactions with cluster N2 were also observed for positions Glu^{89} in the PSST and His^{226}, Arg^{141}, Asp^{143} and Tyr^{144} in the 49 kDa subunit of *Y. lipolytica* complex I, all of which map close to the proximal cluster of [NiFe] hydrogenases. Most of the resistance mutations in the 49 kDa subunit cluster around the region corresponding to the hydrogenase [NiFe] fold. Resistance and hypersensitivity mutations in the PSST subunit are found at various positions, some of which are quite distant from cluster N2 and from the interface with the 49 kDa subunit. It is likely that such mutations exert their effects by altering the secondary structure of the PSST subunit and perhaps even the neighbouring 49 kDa subunit. Mutations that cause inactivity can also be found at some distance from the former [NiFe] centre.

Significantly, mutations in the 49 kDa subunit of *Y. lipolytica* complex I which affect the EPR signature of cluster N2 also produced additional effects like inactivity (Tyr^{144}), resistance (Arg^{141}, Asp^{143}) or hypersensitivity (His^{226}). At position Val^{460} in the 49 kDa subunit of *Y. lipolytica*, corresponding to the hydrogenase [NiFe] centre ligand Cys^{543}, mutations could be generated that displayed all three types of effects.

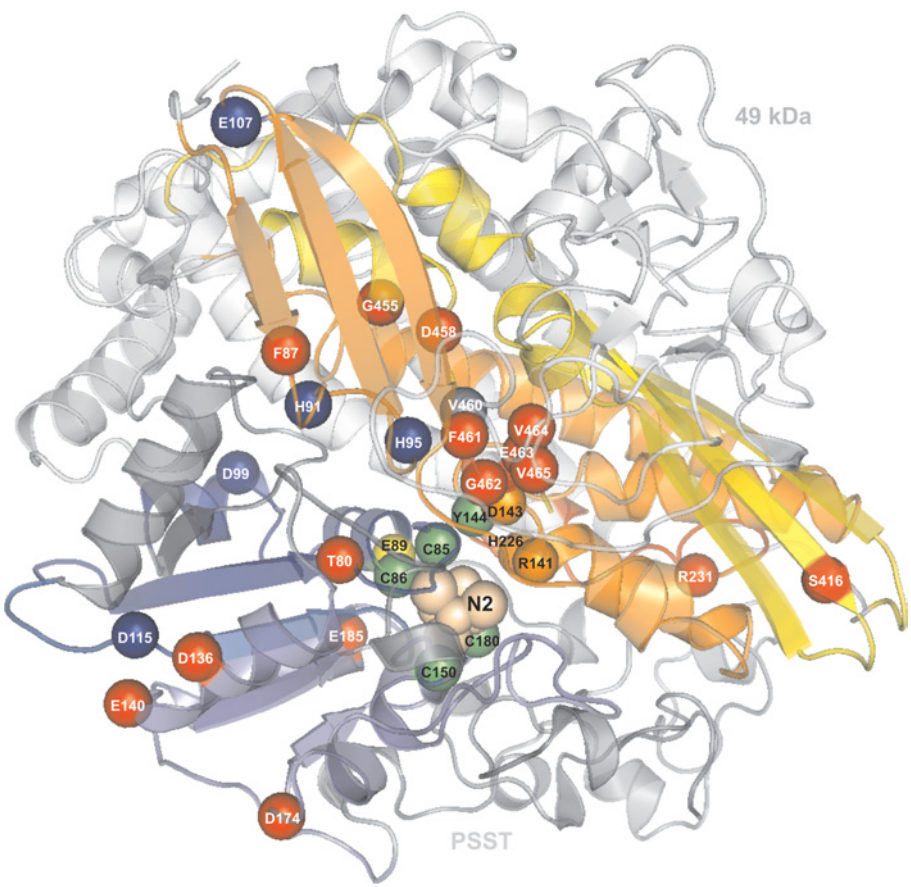
In summary, the observed pattern of effects is fully consistent with the following hypotheses [22,23]: (i) The proximal cluster in the hydrogenase small subunit has evolved into cluster N2 that resides in the PSST subunit, close to the interface with the 49 kDa subunit; and (ii) domains involved in the ligation of the [NiFe] centre in the hydrogenase large subunit have evolved to form a significant part of the ubiquinone reducing catalytic core in complex I.

Localization of the 49 kDa and PSST subunits within complex I

The position of the 49 kDa subunit was determined by electron microscopic single-particle analysis using a monoclonal antibody that recognizes the middle strand of the conserved N-terminal β-sheet [32]. From the finding that this epitope is located close to the distal tip of the peripheral arm, it must be concluded that the ubiquinone reducing catalytic core of complex I is located far away from the surface of the membrane bilayer. To reconcile this unexpected finding with the hydrophobic nature of the substrate ubiquinone, we have postulated that complex I may possess a hydrophobic 'ramp' that enables the substrate to (at least partly) leave the inner mitochondrial membrane bilayer and allows its hydrophilic headgroup to reach the reduction site [32].

Figure 2 | Effects of mutations in the 49 kDa and PSST subunits of *Y. lipolytica* complex I and their positions within the hydrogenase 'map'

Positions within the 49 kDa and PSST subunits of complex I that were targeted by site-directed mutagenesis are highlighted in the X-ray structure of the [NiFe] hydrogenase of *D. fructosovorans* by showing the Cα-atom in space-fill representation. Effects of mutations are colour coded as follows: yellow, alterations of the EPR signature of cluster N2 (shifts in the field position of at least one of the EPR signals, g_z and/or g_{xy}, or strong reduction of signal intensities); red, effects on binding of quinone substrates or quinone-like inhibitors (resistance to benzimidazole in *H. pylori*, or resistance to piericidin A in *Rh. capsulatus*, or elevated K_m for nonylubiquinone or decyl-ubiquinone, or at least 1.5-fold change in IC_{50} for 2-decyl-1-quinazolinyl amine or rotenone); blue, loss of catalytic activity (less than 10% of the original rate); orange, combination of yellow and red; green, combination of yellow and blue; black, combination of yellow, red and blue. Structural elements conserved between both enzyme classes are highlighted in colour (orange, red, and yellow: large subunit; and blue: small subunit), others in light grey. The C-terminal part of the hydrogenase small subunit that is not present in the PSST subunit is shown in dark grey.



Since it appears inevitable that the actual proton pumping device of complex I is contained in the membrane arm and is formed by subunits ND2, ND4 and ND5 which display similarities with bacterial Na^+/H^+ antiporters [33,34], a spatial separation of ubiquinone reduction and proton pumping provides a strong argument for mechanistic models that assume an indirect coupling between these processes in the complex I reaction mechanism (see below).

The proton pumping mechanism of mitochondrial complex I

EPR studies conducted by Ohnishi and co-workers have shown that at least two semiquinone species (Q_{NF} and Q_{NS}, which are approx. 10 and 30 Å (1 Å=10^{-10} m) away from cluster N2 respectively) paramagnetically interact with cluster N2 [35,36]. The redox midpoint potential of cluster N2 (approx. –150 mV in bovine heart [37] and approx. –100 mV in *Y. lipolytica* [23]) is highest among all iron–sulphur clusters in complex I and shows a marked redox-Bohr effect within the physiological pH range [38]. On the basis of these findings, it seemed straightforward to conclude that cluster N2 is involved in proton pumping by complex I.

Again, testing this hypothesis by site-directed mutagenesis in *Y. lipolytica* gave unexpected results. Mutations of His^{226} in the 49 kDa subunit, corresponding to His^{228} in the large subunit of the [NiFe] hydrogenase from *D. fructosovorans* proved especially informative. Mutation $His^{226} \rightarrow M$ shifted the cluster N2 redox midpoint potential to the negative and

rendered it pH independent over the range from pH 6 to pH 8. Unexpectedly however, although the mutation $His^{226} \rightarrow Met$ had apparently removed the cluster N2 associated redox-Bohr group, the mutant enzyme was indistinguishable from the parental one in terms of proton pumping efficiency (K. Zwicker, A. Galkin, S. Dröse, L. Grgic and U. Brandt, unpublished work). Since these results strongly argue against the notion that proton pumping by complex I is connected directly to the redox reaction between cluster N2 and ubiquinone, it seems that the only viable option is assuming a conformational proton pump, operated somehow by ubiquinone intermediates. A conformational change mechanism had also been proposed on the basis of electron microscopic single particle analysis of *E. coli* complex I [11]. However, the data presented so far do not provide sufficient resolution, and X-ray structures of complex I will be required to identify residues and structural folds potentially involved in a conformational pump and test these predictions by site-directed mutagenesis.

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