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Review

Application of the yeast *Yarrowia lipolytica* as a model to analyse human pathogenic mutations in mitochondrial complex I (NADH:ubiquinone oxidoreductase)

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Abstract

While diagnosis and genetic analysis of mitochondrial disorders has made remarkable progress, we still do not understand how given molecular defects are correlated to specific patterns of symptoms and their severity. Towards resolving this dilemma for the largest and therefore most affected respiratory chain enzyme, we have established the yeast *Yarrowia lipolytica* as a eucaryotic model system to analyse respiratory chain complex I. For in vivo analysis, eYFP protein was attached to the 30-kDa subunit to visualize complex I and mitochondria. Deletions strains for nuclear coded subunits allow the reconstruction of patient alleles by site-directed mutagenesis and plasmid complementation. In most of the pathogenic mutations analysed so far, decreased catalytic activities, elevated K_M values, and/or elevated I_{50} values for quinone-analogous inhibitors were observed, providing plausible clues on the pathogenic process at the molecular level. Leigh mutations in the 49-kDa and PSST homologous subunits are found in regions that are at the boundaries of the ubiquinone-reducing catalytic core. This supports the proposed structural model and at the same time identifies novel domains critical for catalysis. Thus, *Y. lipolytica* is a useful lower eucaryotic model that will help to understand how pathogenic mutations in complex I interfere with enzyme function.

Keywords: *Yarrowia lipolytica*; Mutation; Complex I

1. Introduction

Complex I is an essential component of the mitochondrial respiratory chain. As in humans, 7 of the 13 structural genes of the mitochondrial genome code for subunits of complex I; this enzyme is most frequently affected by mtDNA mutations. In recent years, also a growing number of deletions and point mutations that affect nuclear coded subunits of complex I have been described to cause severe degenerative disorders that usually present as Leigh syndrome with or without cardiomyopathy.

While diagnosis and genetic analysis of mitochondrial disorders has made remarkable progress during the past two decades, we still do not understand how a given molecular defect is correlated to a specific pattern of symptoms and their severity. This is partly due to the limited availability of patient samples. However, in the case of complex I, also our knowledge about the structure and function of the affected enzyme is still rather incomplete. One way that may help to overcome these limitations is to reconstruct some of the deleterious mutations in a model organism. Results obtained with animal models have the greatest chance to be directly

Abbreviations: BN-PAGE, blue-native polyacrylamide gel electrophoresis; DBQ, *n*-decylubiquinone; dNADH, deamino-nicotinamide-adenine-dinucleotide (reduced form); DQA, 2-decyl-4-quinazolinyl amine; HAR, hexa-ammine-ruthenium(III)-chloride; I_{50}, inhibitor concentration required for a 50% reduction in catalytic rate; MALDI-TOF MS, matrix-assisted laser desorption/ionisation-time of flight mass spectrometry; NBQ, *n*-nonylubiquinone

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transferable to the pathogenic events in humans. However, genetic manipulations, for example, in mice are much more complicated and time-consuming than in lower eucaryotes like yeast, and therefore animal models are most useful and are applied most efficiently when there is already a profound understanding of the structural and functional basis of the disease under study. Due to the great evolutionary distance, on the other hand, reconstruction of human mutations in a yeast will usually not provide insights that would give immediate answers explaining the complex pathogenic events occurring in a human being. Still, this approach—in particular if combined with broader and thorough structure/function analysis—may be very useful to understand the pathogenic principles that eventually result in a clinical syndrome in humans.

Here we describe the use of the obligate aerobic yeast *Yarrowia lipolytica* as a model organism to study human pathogenic mutations in the nuclear coded subunits of mitochondrial complex I. In many cases, reconstruction of these mutations resulted in specific effects on complex I stability and function. We discuss these results in the light of our model for the ubiquinone-reducing catalytic core of complex I [1,2] and correlate them with patient data compiled from the literature.

2. Complex I in health and disease

Mitochondrial complex I (proton translocating NADH: ubiquinone oxidoreductase, E.C. 1.6.5.3) is the largest and least understood enzyme of the respiratory chain. Its reaction mechanism is still unknown and, in contrast to all other respiratory chain complexes, no X-ray structure is available. Low-resolution structures show an L-shaped molecule, composed of a hydrophilic peripheral arm and a hydrophobic membrane arm [3–6].

Mammalian complex I, exemplified by the well-characterized bovine heart enzyme, contains 46 subunits [7,8]. One molecule of FMN and eight iron–sulfur clusters, only six of which are detectable by EPR spectroscopy [9], serve as redox prosthetic groups. Fourteen "central" subunits are evolutionary conserved between pro- and eucaryotes. The seven central subunits of the peripheral arm that include those that carry all known redox prosthetic groups are encoded by the nuclear genome, while the seven central, highly hydrophobic subunits of the membrane arm are encoded by the mitochondrial genome in mammals and fungi.

Mammalian complex I pumps four protons per two electrons [10,11] and thus generates about 40% of the proton motive force across the respiratory membrane. Since a semiquinone radical is a natural intermediate in their catalytic cycle, complexes I and III are the major production sites for mitochondrial radical oxygen species (ROS), which have been implicated in aging [12]. In recent years, it has been found that mutations in the mitochondrial genome, including mutations in genes for hydrophobic subunits of complex I, are frequent in human cancer cells [13,14]. It has been suggested that such mutations may increase the production of ROS, which in turn could generate somatic mutations.

Poisoning of complex I by prolonged intravenous application of the insecticide rotenone could produce the symptoms of parkinsonism in experimental animals [15,16]. These findings support the view that complex I defects may be central to the pathogenesis of Parkinson's disease [17].

Inherited deficiencies in complex I activity lead to a number of degenerative diseases [18]. In most cases, the clinical presentation is Leigh syndrome (subacute necrotising encephalomyelopathy, MIM 256000), with or without cardiomyopathy. Typically, onset is in early infancy with a progressive course and a fatal prognosis. Diagnostic symptoms include elevated blood lactate levels and characteristic neuropathology with focal, bilateral lesions in one or more areas of the central nervous system, including the brain stem. In most cases, reduced NADH dehydrogenase activity can be observed in crude mitochondria preparations from biopsies or autopsies of muscle or other tissue, or from fibroblast cultures. These materials, however, do not easily allow purification and in vitro characterisation of mutant enzymes.

3. A yeast genetic approach to complex I

Since complex I is absent in fermentative yeasts like *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, we chose the obligate aerobic yeast *Y. lipolytica* as our model system.

Using different approaches, we could successfully identify 37 subunits of *Y. lipolytica* complex I. The genes for the seven central, nuclear coded subunits were cloned using PCR with degenerate primers, followed by screening of a *Y. lipolytica* genomic DNA library [3]. The sequences of the seven central, highly hydrophobic subunits were obtained by completely sequencing the mitochondrial genome [19]. The genes for 23 accessory subunits could be identified in a recently completed draft version of the complete genomic sequence of *Y. lipolytica*. Nineteen of these 23 accessory subunits of *Y. lipolytica* complex I clearly have orthologues in mammalian complex I. Of the remaining four accessory subunits, three appear to be conserved among ascomycetous fungi. One subunit, termed NUWM, was identified for which no homologous sequence could be detected in fungi, mammals, or any other organism using standard BLAST searches.

After separation of *Y. lipolytica* complex I using doubled SDS-PAGE [20], we counted 39 protein spots, which is only slightly higher than the number of 37 subunit genes identified in the genome. We therefore estimate that about 90% of the total subunit inventory of *Y. lipolytica* complex I is known to date. Twenty-three nuclear coded proteins, including all seven central and 15 accessory subunits of the peripheral arm,

could be assigned to protein spots in doubled SDS gels using MALDI-TOF MS of tryptic fragments [21].

Besides complex I, mitochondria from plants, fungi and many bacteria also contain one or several alternative NADH dehydrogenases [22]. These enzymes consist of a single subunit and contain FAD as sole redox prosthetic group. They carry out the same reaction as complex I but are unable to pump protons across the respiratory membrane. Alternative enzymes from eucaryotes can have either external or internal orientation, i.e., their active site can face either the cytosolic or the matrix side of the inner mitochondrial membrane. We have shown that in mitochondria from *Y. lipolytica*, only a single, external alternative enzyme (NDH2) is active [23]. By N-terminal attachment of the mitochondrial import sequence from the 75-kDa subunit of *Y. lipolytica* complex I, an internal version was constructed. Interestingly, the internal, but not the external, version of NDH2 was able to compensate for the NADH dehydrogenase activity of complex I [24]. Only after the gene for the internal version of NDH2 had been integrated into the *Y. lipolytica* genome it became possible to generate haploid strains in which genes for nuclear coded subunits of complex I had been individually deleted. These strains could then be complemented by site-directed mutant versions carried on replicative plasmids.

Attachment of a hexa-histidine tag to the C-terminus of the 30-kDa subunit allowed fast and efficient purification of complex I from dodecyl maltoside-extracted mitochondrial membranes [25].

To be able to monitor potential effects of complex I mutations on mitochondrial morphology in live cells, the eYFP variant (BD Clontech) of the green fluorescent protein was inserted between the C-terminus of the 30-kDa subunit and the hexa-histidine tag. When plasmid pEYFP30 that carried this construct was introduced into haploid *Y. lipolytica* strains, green fluorescence of mitochondria could be observed using confocal laser scanning microscopy (Fig. 1A). *Y. lipolytica* mitochondria

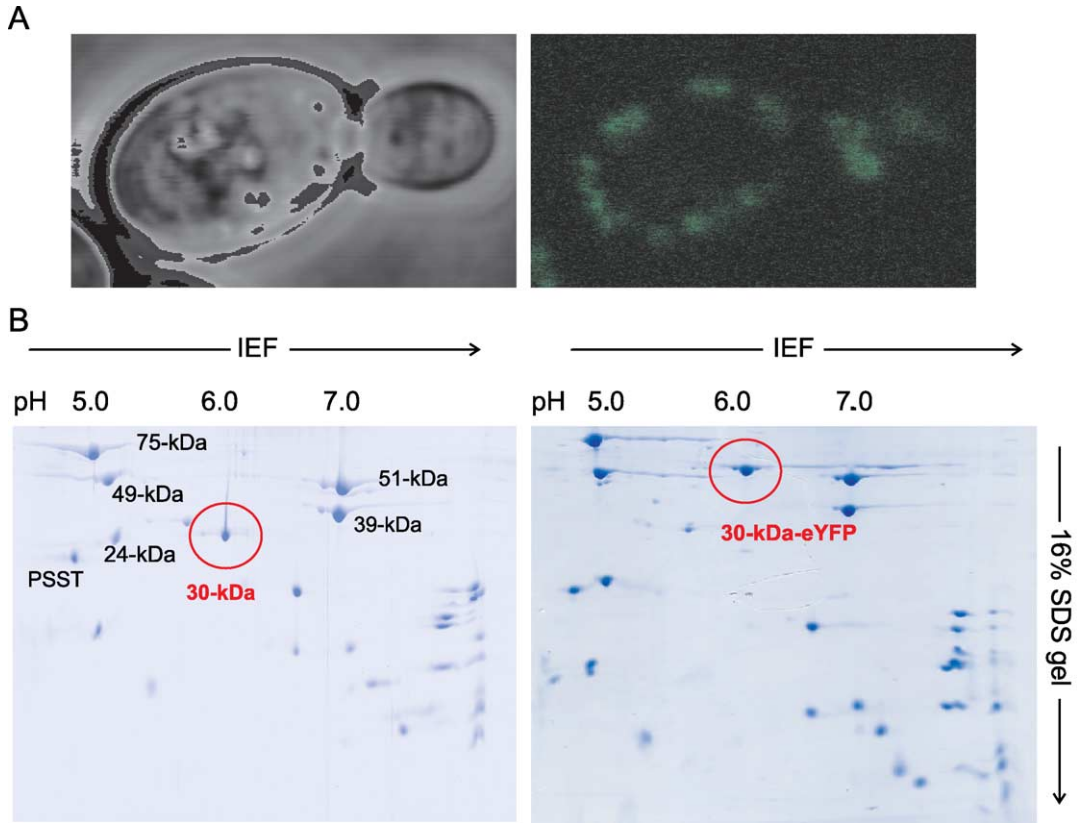

Fig. 1. In vivo labelling of complex I by eYFP. An in-frame *Nde*I site between the 3′ end of the 30-kDa subunit open reading frame and the hexa-histidine tag sequence was created by PCR using primers 5′-GGCTGCGGCCATATGCTTCTTGTCGCCTTCCTTCTCCTC-3′ (binds in antisense orientation to the C-terminal end of the 30-kDa subunit open reading frame) and 5′-GCCGCAGCCCATCATCATCATCATCACTAG-3′ (binds in sense orientation to the hexa-histidine tag), followed by self-ligation of the PCR product. The gene for eYFP (containing amino acid substitutions S65G, V68L, S72A and T203Y) was amplified by PCR as a 0.7-kb *Nde*I fragment using primers 5′-ATATATCATATGGTAAGCAAGGGCGAGG-3′ and 5′-ATATATCATATGCTTGTACAGCTCGTCCAT-3′ and cloned into this *Nde*I site, resulting in plasmid pEYFP30. (A) Visualisation of *Y. lipolytica* mitochondria using eYFP. A1, Phase contrast; A2, Confocal laser scanning micrograph of cells from strain Δ*nugm* GH1 (*nugm*::*URA3*, *his1*$^{-}$, *ura3*$^{-}$, *leu2*$^{-}$, *xpr2*$^{-}$, *Mat*A) complemented with plasmid pEYFP30. (B) Detection of the 30 kDa–eYFP fusion protein by 2D (IEF/SDS) PAGE. Four-hundred micrograms of purified complex I was separated in the first dimension by IEF using the IPGphor system (Amersham Pharmacia Biotech) and the 13-cm immobilised pH gradient (IPG) strips (pH 3–10) and by 16% SDS PAGE in the second dimension. B1, Complex I from strain PIPO (*NUGM*-Ht2, *lys11*$^{-}$, *ura3*$^{-}$, *leu2*$^{-}$, *xpr2*$^{-}$, *Mat*A). The 30-kDa subunit is circled in red. B2, Complex I from strain Δ*nugm* GH1 complemented with plasmid pEYFP30. A novel band of about 53-kDa that presents the 30-kDa subunit fused with eYFP is circled in red.

were found to be spherical to ovoid in shape and distributed in the cytoplasmic rim between the central vacuole and the cell wall. The eYFP-labelled version of complex I could also be purified by Ni^{2+} affinity chromatography without any modification to the standard protocol [25]. The altered size of the 30 kDa–eYFP protein could be demonstrated using 2D (IEF/SDS) PAGE (Fig. 1B).

4. The catalytic core of complex I

The reaction mechanism of complex I is still not understood [26]. Electrons from NADH are transferred via noncovalently bound FMN, noncovalently bound to the 51-kDa subunit, and several bi- and tetranuclear iron–sulfur clusters. There is general agreement that tetranuclear cluster N2, as the last step in this redox chain, is the immediate electron donor to ubiquinone. Site-directed mutagenesis in *Y. lipolytica*, *Neurospora crassa* and *Escherichia coli* [27–30] has demonstrated that N2 is located in the subunit homologous to bovine PSST.

It has been known for a long time that some subunits of complex I are homologous to subunits of [NiFe] hydrogenases [31,32], H_2-producing or H_2-consuming enzymes found both in archaebacterial and eubacterial species [33]. The 49-kDa and PSST subunits of bovine heart complex I are related to the large and small subunits of water-soluble [NiFe] hydrogenases, respectively. The PSST homologous subunits of membrane-bound [NiFe] hydrogenases and of complex I have suffered a C-terminal deletion that removed two out of three iron–sulfur cluster binding motifs. A novel, ferredoxin-like subunit (corresponding to subunit TYKY of bovine heart complex I) has been acquired instead [34].

Assuming that the structural fold has been largely conserved during evolution, we proposed that the known structures of water-soluble [NiFe] hydrogenases like that from *Desulfovibrio fructosovorans* [35] could be used as models for the 49-kDa and PSST subunits of complex I. When the effects of point mutations in the 49-kDa subunits from *Rhodobacter capsulatus* [36,37] and from *Y. lipolytica* [1] were compared with their positions in this "hydrogenase model", two striking observations could be made that strongly support this assumption: Mutations that result in reduced affinity for quinone-like inhibitors map close to the region corresponding to the hydrogenase [NiFe] site, while mutations that affect the EPR signature of cluster N2 map close to the subunit interface where the large subunit of hydrogenase is in contact with the proximal iron–sulfur cluster held by the small subunit. We concluded that a significant part of the ubiquinone binding pocket of complex I is provided by the 49-kDa subunit and has evolved from the [NiFe] site in the hydrogenase large subunit and that cluster N2 has evolved from the proximal iron–sulfur cluster in the hydrogenase small subunit [1,2].

5. Pathogenic mutations reconstructed in *Y. lipolytica*

5.1. 49-kDa subunit

Three mutations in the 49-kDa subunit of complex I (Fig. 2) had been described in patients affected with cardiomyopathy and encephalomyopathy [38], namely R228Q, P229Q and S413P. All three residues are conserved in *Y. lipolytica* and the mutations were remodelled by introducing substitutions R231Q, P232Q and S416P.

Characterisation of all mutants which were sufficiently active was routinely carried out using the following methods: (i) BN-PAGE analysis of mitochondrial membranes as a qualitative test for complex I assembly; (ii) measuring the electron transfer from NADH to nonphysiological acceptor HAR, which provides a more quantitative estimate of the complex I of mitochondrial membranes; (iii) measuring the inhibitor sensitive catalytic activity from NADH to DBQ, a hydrophobic analogue of the physiological acceptor ubiquinone; (iv) determining the concentration required for halfmaximal inhibition of NADH:DBQ activity by two inhibitors of complex I. These data are presented in Table 1.

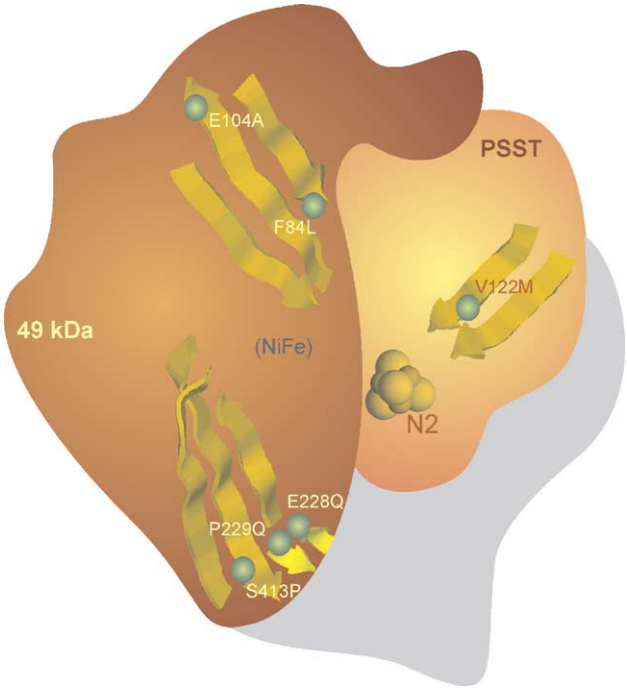

Fig. 2. Position of human pathogenic mutations relative to the catalytic core of complex I. The 49-kDa and PSST homologous subunits of complex I and structural elements within them were modelled according to the structure of the water-soluble [NiFe] hydrogenase from *D. fructosovorans*. The approximate locations of the point mutations are marked in green and numbered according to the human subunits. Cluster N2 in the PSST subunit, which corresponds to the proximal iron–sulfur cluster of hydrogenases, and the region in the 49-kDa, which corresponds to the [NiFe] site of hydrogenases, are indicated. The part of the small hydrogenase subunit that is not present in the PSST subunit is depicted in light grey.

Table 1
Reconstruction and characterisation of human pathogenic complex I mutations in the yeast *Y. lipolytica*

Subunit	Human mutation				Reconstruction in *Y. lipolytica*						
	Position	CI activity (%)[a]	Death at	Reference	Position	CI content (%)[b]	CI activity (%)[c]	K_M (μM)[d]	I_{50} (DQA) (nM)[e]	I_{50} (Rotenone) (nM)[f]	Reference
49 kDa	R228Q	57	2 years	Loeffen et al., 2001	R231Q	84	101	16	17	900	This work
					R231E	112	74	29	20	850	
	S413P	69	3 years	Loeffen et al., 2001	S416P	90	111	17	17	1100	
					S416A	109	86	39	19	1500	
	P229Q	24	4 days	Loeffen et al., 2001	P232Q	38	17	n.d.	n.d.	n.d.	
					P232G	67	56	7	23	500	
	F84L[g] E104A[g]	20, 46	3.5 yrs, –	Smeitink et al., in preparation	F87L E107A	130 140	59 0	21 n.d.	50 n.d.	600 n.d.	
PSST	V122M	19	5 years	Triepels et al., 1999	V119M	110	55	12	10	700	Ahlers et al. [43]
TYKY	P79L[g] R102H[g]	39	11 weeks	Loeffen et al., 1998	P98L R121H	60 80	57 45	12 19	20 10	700 500	Ahlers et al. [43]
30 kDa	T145I[g] R199W[g]	27	10 years	Bénit et al., 2004	T157I R211W	85 83	87 90	12 14	25 20	500 500	this work

n.d.=not determined.

[a] Residual activity in muscle tissue biopsies or autopsies, for each patient described in sufficient detail, expressed in % of the lowest reference value.

[b] Measured as (nonphysiological) NADH:HAR activity of mitochondrial membranes. 100% values observed in parental strains were 1.0 U (PSST), 1.0 U (TYKY), 0.7 U (30 kDa) and 1.0 U (49 kDa) per mg of total protein in the assay.

[c] Measured as dNADH:NBQ activity (PSST and TYKY mutations) or dNADH:DBQ activity (other mutations) of mitochondrial membranes, normalized by content. 100% values observed in parental strains were 0.40 U (PSST), 0.44 U (TYKY), 0.53 U/(30 kDa) and 0.30 U (49 kDa) per mg of total protein in the assay.

[d] K_M (NBQ) for PSST and TYKY mutations, K_M (DBQ) for all other mutations. Determined using the program Enzfitter (Biosoft, Cambridge, England). Values in parental strains were in the range of 14 to 25 μM.

[e] Values in parental strains were in the range of 13 to 25 nM.

[f] Values in parental strains were in the range of 500 to 600 nM.

[g] Compound heterozygous patient(s).

Mutants R231Q and S416P, as judged from NADH:HAR activities and BN-PAGE analyses of mitochondrial membranes (data not shown), had virtually normal amounts of fully assembled complex I. Also, specific dNADH:DBQ activities, K_M values for DBQ and I_{50} values for the specific complex I inhibitor DQA were almost the same as in the parental strain. Only the I_{50} values for rotenone were found to be 1.5–2 times higher in these mutants, indicating slight resistance to this inhibitor. This suggests a subtle effect on the ubiquinone binding pocket of complex I. Purified complex I from mutant R231Q displayed normal EPR spectra, could be reactivated by the addition of phospholipids and exhibited proton pumping after reconstitution into proteoliposomes (data not shown). To explore the possibility that mutations R231Q and S416P had made complex I less stable, dNADH:DBQ activities were checked after incubation of mitochondrial membranes for 10 min at different temperatures. Again, no difference to the wild-type strain could be detected. From these results, it was unclear why these mutations are pathogenic in humans. Therefore, the more drastic mutations R231E and S416A were created. In mutant R231E, there was no effect on complex I content. Its dNADH:DBQ activity was slightly reduced and its K_M value appeared to be somewhat elevated. Mutant S416A had regular complex I content and dNADH:DBQ activity was only slightly reduced. K_M and the I_{50} values for rotenone were both significantly higher than in the parental strain. Overall, these results seemed to suggest that the conserved arginine and proline are in a position within the 49-kDa subunit that is peripheral or indirectly linked to the ubiquinone-reducing core of complex I.

Mitochondrial membranes from mutant P232Q had less than 40% NADH:HAR activity as compared to the parental strain. Using BN-PAGE of mitochondrial membranes, a complex I-specific band could not be detected (data not shown). Both results indicate that complex I assembly was severely impaired in this mutant. dNADH:DBQ activity was less than 20% of the normal value. Due to this low activity, determination of K_M for DBQ and I_{50} values for DQA and rotenone was not possible. However, when the more conservative mutation P232G was introduced, complex I was assembled normally as judged by BN-PAGE of mitochondrial membranes (data not shown). Its content was reduced by more than 30% and complex I-specific dNADH:DBQ activity was only 50% of that of the parental strain. Thus, consistent with the typical role of a conserved proline, this residue seemed to be critical for proper folding of the 49-kDa subunit and assembly of complex I.

Recently, two additional point mutations in the NDUFS2 gene, resulting in substitutions F84L and E104A, were identified in two compound heterozygous Leigh patients from the same family (J. Smeitink et al., in preparation).

These were reconstructed at the corresponding conserved positions in the *Y. lipolytica* 49-kDa subunit (F87L and E107A). In mitochondrial membranes from mutant F87L, complex I content was normal, while specific dNADH:DBQ activity was reduced to about 60% of the parental strain value. The K_M value for DBQ and the I_{50} value for rotenone were normal, but a twofold resistance towards DQA was observed. Strikingly, mutant E107A, while having at least the same complex I content as the parental strain, displayed no dNADH:DBQ activity at all, suggesting that this glutamate is critical for the catalytic activity of complex I.

Since the 49-kDa subunit makes a significant contribution to the ubiquinone-reducing catalytic core of complex I, it is of particular interest to correlate the effects of Leigh syndrome mutations with their positions in the [NiFe] hydrogenase structural model (Fig. 2). It has been shown previously that mutations within those segments of the 49-kDa subunit sequence that align with the four structural elements that come close to the [NiFe] site in the *D. fructosovorans* hydrogenase structure either have marked effects on the affinity for DBQ and/or quinone-like inhibitors or affect the EPR signature of cluster N2, located in the neighboring PSST subunit [2].

One of these structural elements, which is likely to be conserved between [NiFe] hydrogenases and complex I, is the loop that is bounded by conserved glycines (G224, G235 and G236 in the *D. fructosovorans* large subunit and G219, G230 and G231 in the *Homo sapiens* 49-kDa subunit) and carries a conserved histidine at its tip (H228 in the *D. fructosovorans* large subunit and H223 in the *H. sapiens* 49-kDa subunit). Mutations R228Q and P229Q (*H. sapiens* numbering) are contained within this segment, but the affected residues correspond to positions at the base of this loop that is farthest away from the [NiFe] site. In the hydrogenase structure, this base is rooted in a small, antiparallel β-sheet, located in the region of the large subunit that is juxtaposed to that part of the small subunit which is not present in the PSST subunit.

A second structural element, which is likely to be conserved between [NiFe] hydrogenases and complex I, contains a conserved proline (P475 in the *D. fructosovorans* large subunit and P403 in the *H. sapiens* 49-kDa subunit). In the hydrogenase structure, this loop is connected to a β-strand that points away from the [NiFe] site and forms the middle part of a more extended, three-stranded anti-parallel β-sheet. Again, this structural element of the large hydrogenase subunit is juxtaposed to that part of the small subunit which is not present in the PSST subunit. Mutation S413P (*H. sapiens* numbering) is contained in this β-sheet, but again, with respect to the [NiFe] site, the affected residue is found at its distal end.

The region around positions F84 and E104 in the *H. sapiens* 49-kDa subunit aligns with a three-stranded β-sheet that is found at the N-terminal end in the *D. fructosovorans* [NiFe] hydrogenase large subunit structure. As has been described before [39], the PROF algorithm [40] predicts a similar secondary structure for the corresponding parts of the 49-kDa subunits from various organisms. Since sequence similarity is low in this region, it is not easy to say exactly which residues in the *D. fructosovorans* large subunit correspond to mutated residues in the *H. sapiens* 49-kDa subunit, but it is likely that F84 is situated immediately downstream of the first β-strand and E104 immediately upstream of the third β-strand. Remarkably, in this region two conserved histidines (H91 and H95 in *Y. lipolytica*) were found to be critical for catalytic activity of complex I [41]. This supports the notion that this part of the 49-kDa subunit is of central importance for the function of complex I.

In summary, all Leigh syndrome mutations in the 49-kDa subunit described here are predicted by the hydrogenase model to reside on the surface of the subunit. Consistent with this position, some of them (R231Q and S416P in *Y. lipolytica*) showed only small and indirect effects, while others (P232Q and E107A in *Y. lipolytica*) had dramatic effects on catalytic activity. The effects of mutations in the protein periphery may result from distortion of domains that are then transmitted to the ubiquinone-reducing catalytic core by domain–domain interactions within the 49-kDa subunit. However, it is tempting to speculate that the residues affected by Leigh syndrome mutations may also be involved in interactions of the 49-kDa subunit with neighboring complex I subunits.

5.2. PSST subunit

Homozygosity for a point mutation in *NDUFS7*, resulting in the substitution in the PSST subunit of a conserved valine for methionine (V122M), had been found in two brothers affected with Leigh syndrome [42]. This mutation was reconstructed at the corresponding position (V119M) in the PSST homologous subunit of *Y. lipolytica*. While complex I content of the mutant strain was the same as in the parental strain, catalytic activity was reduced by about 50% [43].

Valine 119 in the *Y. lipolytica* PSST subunit corresponds to a conserved valine in [NiFe] hydrogenases. This valine (V74 of the small subunit of the *D. fructosovorans* enzyme) is located within a parallel β-strand that is part of a β-sheet structure which stabilizes the domain bearing the proximal iron–sulfur cluster. Since it is not known to which extent this β-sheet is conserved between [NiFe] hydrogenases and complex I, only the central two of its four β-strands are shown in Fig. 2. Consistent with its position in the "hydrogenase model", mutation V119M affected the EPR signature of iron–sulfur cluster N2 (Fig. 3). When compared to the spectrum of the parental strain, the N2 signal appeared to be reduced in intensity.

5.3. TYKY subunit

Two point mutations in *NDUFS8*, resulting in substitutions P79L and a R102H, had been identified in a

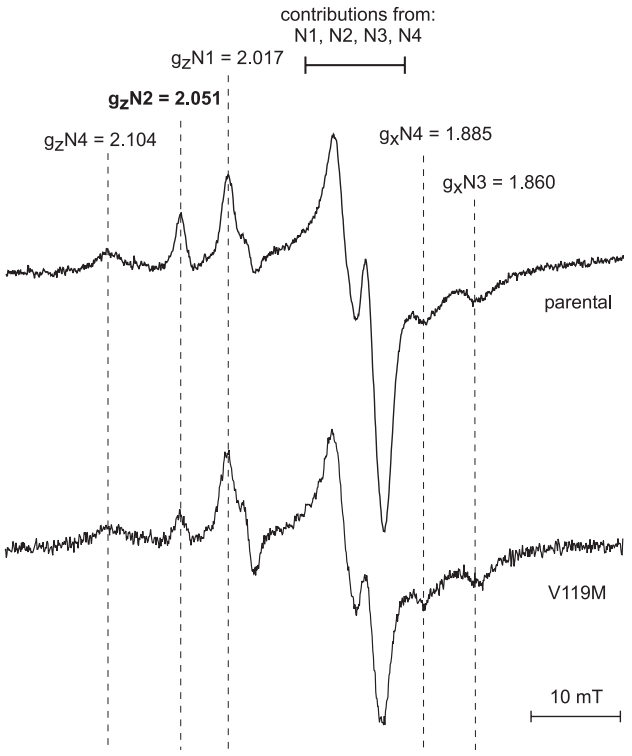

Fig. 3. EPR spectra of complex I from parental strain and mutant V119M. Contributions of individual clusters are indicated together with their characteristic g values. Spectra were obtained on a Bruker ESP 300E spectrometer equipped with a liquid helium continuous flow cryostat, ESR 900 from Oxford Instruments. Samples of purified complex I were mixed with NADH in the EPR tube and frozen after 30-s reaction time. Spectra were recorded at 12 K with the following instrument settings: Microwave frequency 9.47 GHz, microwave power 2 mW, modulation amplitude 0.8 mT.

compound heterozygous patient with neuropathologically proven Leigh syndrome [44]. When these mutations were reconstructed in *Y. lipolytica* (P98L and R121H), reduced catalytic rates, again in the range of 50% of the parental strain value and somewhat reduced complex I contents, could be observed [43]. Both mutations lie upstream of the highly conserved sequence CXXCXXCXXXCP which is the first half of a ferredoxin-type binding motif for the coordination of two tetranuclear iron–sulfur clusters [45]. However, since these clusters are EPR silent, it is unknown whether the Leigh syndrome mutations have an influence on their geometry.

5.4. 30-kDa subunit

Two point mutations in the 30-kDa subunit have been reported to cause Leigh syndrome [46]. They result in amino acid substitutions T154I and R199W. Both mutations were reconstructed in *Y. lipolytica* (T157I and R211W), and complex I activity was characterized in mitochondrial membranes. However, no significant alterations in complex I content or activity could be observed in isolated mitochondrial membranes.

5.5. 24-kDa subunit

An exon 2 skipping mutation in the human *NDUFV2* gene (IVS2+5_+8delGTAA) had been found to cause hypertrophic cardiomyopathy and encephalomyopathy [47]. It removes part of the mitochondrial targeting sequence and the processing site from the 24-kDa subunit precursor. Analysis of patient mitochondria, however, indicated that complex I assembly and activity were only reduced, but not completely abolished. Therefore, it seemed likely that mitochondrial import and assembly into functional enzyme can occur without proteolytic processing of the 24-kDa subunit precursor, but this could not be demonstrated using biopsies or cell cultures derived from patients. We have generated a similar mutation in the orthologous NUHM protein from *Y. lipolytica* (Kerscher et al. submitted). The presequences of the 24-kDa subunits from *H. sapiens* and from *Y. lipolytica* are both rich in aliphatic and basic residues and have the ability to form amphipathic helices but display only weak similarities on the sequence level. Furthermore, while the *Y. lipolytica* presequence contains a −3 Arg processing signal and is presumably processed by mitochondrial processing peptidase (MPP) in a single step, the presence of a −10 Arg residue in the human presequence indicates a two-step processing by the sequential action of MPP and the mitochondrial intermediate peptidase MIP [48,49]. We created deletion *NUHM*Δ17–32, which removes those residues that align with the residues encoded by the second exon of *NDUFV2*. When the *NUHM*Δ17–32 allele was introduced into a *nuhm*Δ strain, we could show that complex I content and activity of mitochondrial membranes isolated from the resulting strain were almost identical to the parental strain values. The molecular mass of the mutant subunit was determined using MALDI-TOF MS, yielding a value that was consistent with the hypothesis that the presequence remnant had been retained on the mature subunit. Since the isoelectric point was shifted and the N-terminus was blocked for Edman degradation, it is likely that the initiator methionine was acetylated. These results demonstrated that removing the processing signal from the precursor of the 24-kDa subunit prevented the cleavage of the import sequence but not the proper assembly of complex I. We concluded that the amino-terminus of this subunit is likely to reside on the surface of the multiprotein complex. With regard to the pathogenic mutation in humans, our data explain why a significant amount of assembled complex I was found in patient mitochondria.

6. Conclusions

The function of complex I is essential for aerobic metabolism. Therefore, it can be assumed that human Leigh syndrome mutations, although resulting in severe, usually

fatal clinical phenotypes, cause only moderate defects on the enzyme level.

Reconstruction of pathogenic mutations in complex I of *Y. lipolytica* provides an excellent system for biochemical studies on complex I, either in its natural environment, in mitochondrial membrane, or in purified form. Notably, all pathogenic point mutations reported so far affect residues that are conserved in the yeast sequences. As summarized here, reduced stability of the multiprotein enzyme, reduced catalytic activity and/or altered affinities for the substrate ubiquinone and/or for quinone-like inhibitors could be observed in many cases in the *Y. lipolytica* model system.

Despite the large evolutionary distance between humans and yeasts, it is of interest to correlate the *Y. lipolytica* data with patient data reported in the literature: Although it is impossible to reach definite conclusions with the limited data set available so far, at least in the cases summarized here, the extent of the functional defects observed in the *Y. lipolytica* mutants correlates remarkably well with the life span of patients affected by the same mutations (Table 1). In fact, this correlation seems to be better than with the complex I activities reported for the patient tissues. This may reflect on the difficulties in judging the severity of complex I defects in individual patients by in vitro activity testing, a matter that has been given careful consideration [50,51].

On the other hand, it should be stressed that when using a unicellular organism, it is inherently impossible to study the impact of complex I mutations on highly integrated aspects of human biology. Such questions include the striking organ specificity that is observed with many point mutations in complex I, and the potential role of complex I defects in tumorigenesis. However, the *Y. lipolytica* model may offer an attractive possibility to test the hypothesis that hypertrophic cardiomyopathies arise as a consequence of increased ROS production. It will be interesting to find out whether increased ROS production can be detected in some of the Leigh mutant versions of complex I after reconstitution in *Y. lipolytica*.

Many fundamental questions on the reaction mechanism of complex I are still open. Reconstruction of pathogenic mutations in *Y. lipolytica* highlights important regions within complex I subunits and will help to promote basic research on complex I that eventually will provide a better understanding of how the pathogenic mutations interfere with enzyme function on a molecular level.

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