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# Application of the obligate aerobic yeast *Yarrowia lipolytica* as a eucaryotic model to analyse Leigh syndrome mutations in the complex I core subunits PSST and TYKY

Pamela M. Ahlers, Aurelio Garofano, Stefan J. Kerscher, Ulrich Brandt *

*Universitätsklinikum Frankfurt, Institut für Biochemie I, ZBC, Theodor-Stern-Kai 7, Haus 25B, D-60590 Frankfurt am Main, Germany*

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**Abstract**

We have used the obligate aerobic yeast *Yarrowia lipolytica* to reconstruct and analyse three missense mutations in the nuclear coded subunits homologous to bovine TYKY and PSST of mitochondrial complex I (proton translocating NADH:ubiquinone oxidoreductase) that have been shown to cause Leigh syndrome (MIM 25600), a severe progressive neurodegenerative disorder. While homozygosity for a V122M substitution in NDUFS7 (PSST) has been found in two siblings with neuropathologically proven Leigh syndrome (R. Triepels et al., Ann. Neurol. 45 (1999) 787), heterozygosity for a P79L and a R102H substitution in NDUFS8 (TYKY) has been found in another patient (J. Loeffen et al., Am. J. Hum. Genet. 63 (1998) 1598). Mitochondrial membranes from *Y. lipolytica* strains carrying any of the three point mutations exhibited similar complex I defects, with $V_{max}$ being reduced by about 50%. This suggests that complex I mutations that clinically present as Leigh syndrome may share common characteristics. In addition changes in the $K_m$ for *n*-decyl-ubiquinone and $I_{50}$ for hydrophobic complex I inhibitors were observed, which provides further evidence that not only the hydrophobic, mitochondrially coded subunits, but also some of the nuclear coded subunits of complex I are involved in its reaction with ubiquinone. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Mitochondria; Complex I; Yeast; *Yarrowia lipolytica*; Leigh syndrome; Point mutation

## 1. Introduction

Complex I, the first enzyme of the respiratory chain, is also the largest and least understood one. In mammals, it has a $M_r$ of almost 1000 and consists of 43 different subunits [1], seven of which are encoded by the mitochondrial genome. Its size and complexity has proven a major obstacle to the analysis of complex I. Since a semiquinone radical is a natural intermediate in the electron transfer reaction from NADH to ubiquinone, it is also an important source of reactive oxygen species (ROS) [2]. Consequently, a large number of genetic disorders are caused by mutations in complex I. While the role of mtDNA mutations [3,4] as a major contributor to complex I deficiency (MIM 252010) has been widely recognised, recent findings have shown that mutations in nuclear encoded subunits of complex I are also fairly common among the group of congenital OXPHOS defects and can result in diverse path-

Abbreviations: dNADH, deamino-NADH; HAR, hexamine-ruthenium(III)-chloride; DBQ, 2-*n*-decyl-ubiquinone; DQA, 2-decyl-4-quinazolinyl amine

* Corresponding author. Fax: +49 (69) 63016970; E-mail: brandt@zbc.klinik.uni-frankfurt.de

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ologies. In a group of 60 patients with isolated complex I defects described by Robinson, the most common clinical presentation, however, was Leigh syndrome with or without cardiomyopathy [2]. Leigh syndrome, or subacute necrotising encephalomyelopathy, is a severe, progressive neurodegenerative disorder with an estimated incidence of about 1:40 000 in live births. It is a genetically heterogenous entity which may display autosomal recessive inheritance caused by defects in the genes for biotinidase [5], pyruvate dehydrogenase complex (PDHc), OXPHOS complex II [6] or IV [7], X-linked inheritance caused by defects in the $E_{1\alpha}$ subunit of the PDHc [8], or maternal inheritance associated with defects in subunit 6 of ATP synthetase [9,10], tRNA(Lys) [11], tRNA(Trp) [12], tRNA(Val) [13], tRNA(Leu) [14] and MERFF mutations A8344G [15] and A3242G [16].

Recently, researchers at the Nijmegen Center for Mitochondrial Disorders (Nijmegen, The Netherlands) have identified point mutations in genes for subunits of complex I as the causative genetic defects in two cases of neuropathologically proven Leigh syndrome that resulted in death in early infancy. One patient was found to carry a homozygous G364A transition in the *NDUFS7* gene encoding the homologue of the PSST subunit of bovine heart complex I [17], resulting in a V122M substitution. Another patient was compound heterozygous for two transitions (C236T, resulting in P79L and G305A, resulting in R102H) in the *NDUFS8* gene encoding the homologue of the TYKY subunit of bovine heart complex I [18]. All three mutations were completely recessive, since no clinical symptoms could be detected in the heterozygous parents. NADH:ubiquinone-1 oxidoreductase activity was low in all tissues examined, suggesting that a reduction in the electron transfer and/or proton pumping activities of complex I can cause Leigh syndrome.

The intriguing finding that the three nuclear Leigh syndrome point mutations affecting complex I described so far reside in central complex I subunits of unclear catalytic function and involve residues that are fully conserved among eucaryotes prompted us to reconstruct them in the obligate aerobic yeast *Yarrowia lipolytica* by site-directed mutagenesis of the genes homologous to human *NDUFS7* and *NDUFS8*. We have recently established *Y. lipolytica* as a new model system for the analysis of complex I [19]. This organism combines the powerful tools of yeast genetics [20] with the possession of a respiratory chain complex I that is stable enough to allow straightforward purification (Djafarzadeh Andabili et al., submitted). This approach allows the in vitro characterisation of mutant forms of complex I. In particular, we were interested in the case of the two compound heterozygous mutations in *NDUFS8*, where the relative contribution of the two different mutant forms to total complex I activity was unclear.

## 2. Materials and methods

### *2.1. Substrates and inhibitors*

*n*-Decyl-ubiquinone (DBQ), deamino-NADH (dNADH), hexamine-ruthenium(III)-chloride (HAR) and rotenone were purchased from Sigma. DQA was a generous gift from Aventis CropScience, Frankfurt am Main.

### *2.2. Strains*

*Y. lipolytica* strains E129 (*Mat*A, *lys11*-23, *ura3*-302, *leu2*-270, *xpr2*-322) and E150 (*Mat*B, *his*-1, *ura3*-302, *leu2*-270, *xpr2*-322) were a kind gift from Prof. C. Gaillardin, INRA, Paris, France. The diploid strain GB1 was produced by mating E129 and E150. *Y. lipolytica* genetic techniques were carried out according to [20].

### *2.3. Deletion of NUKM and NUIM, the Y. lipolytica homologues of PSST and TYKY*

The isolation of the *Y. lipolytica NUKM* and *NUIM* genes (Djafarzadeh Andabili et al., submitted) and the deletion of the *NUKM* gene by homologous recombination with a *LEU2* marked deletion allele [19] have been described previously. The *NUIM* gene was deleted by homologous recombination with a *LEU2* marked deletion allele as follows: a 2.6 kb *Sal*I fragment from genomic clone A3 was digested with *Bam*HI and partially with *Pst*I, removing the *NUIM* open reading frame (ORF) except for the first 20 and last ten amino acids. Into this 0.6 kb gap the *Y. lipolytica LEU2* marker gene was inserted

in opposite orientation to the original *NUIM* ORF as a 1.9 kb PCR product, amplified using primers containing the appropriate restriction sites. The resulting 4.0 kb fragment was isolated and used for transformation of diploid *Y. lipolytica* cells (genotype: *his*+/−, *lys*+/−, *leu2*/*leu2*, *ura3*/*ura3*). Leucine prototrophs were checked for homologous recombination by Southern blotting and PCR using combinations of outward primers derived from the *LEU2* sequence and inward primers derived from genomic sequences flanking the fragment used for transformation.

### 2.4. Site-directed mutagenesis of Y. lipolytica

A 2.8 kb *Hin*dIII partial fragment containing the complete *NUIM* gene or a 2.3 kb *Eco*RI fragment containing the complete *NUKM* gene were subcloned into the *Y. lipolytica* replicative plasmid pINA443 which carries the *Y. lipolytica URA3* marker gene. Point mutations were created using the QuikChange Site-Directed Mutagenesis Kit from Stratagene. Mutated plasmids were confirmed by DNA sequencing and used for transformation of the corresponding heterozygous deletion strain. Haploids carrying the chromosomal deletion and the plasmid-borne mutant allele were isolated after sporulation by their prototrophy for leucine and uracil. *LEU2*, *URA3* spores were tested for absence of the genomic copy of *NUIM* or *NUKM* by PCR or Southern blotting and the mutation was reconfirmed by sequencing of the entire open reading frame on the plasmid.

### 2.5. Functional analysis

Unsealed mitochondrial membranes were isolated as described previously [21]. dNADH:DBQ oxidoreductase activity which is specific for complex I was assayed at 30°C essentially as described [21] with 50 μg/ml unsealed mitochondrial membranes, 100 μM dNADH and 60 μM DBQ in a buffer containing 50 mM Tris-HCl, pH 7.4 and 2 mM KCN. NADH:HAR activity which only relies on a functional 51 kDa subunit and is specific for complex I was used as a measure for complex I content [19] and assayed at 200 μM NADH and 2 mM HAR in 20 mM $Na^+$/HEPES, pH 8.0 and 2 mM KCN at 30°C. Michaelis-Menten parameters were determined by varying the concentration of DBQ (0–100 μM). $I_{50}$ values for DQA and rotenone were determined by adding the inhibitor from an ethanolic stock solution to the proceeding reaction. Data were analysed using the ENZFITTER programme (Elsevier).

## 3. Results

Cloning of the genes for the seven nuclear coded subunits of complex I that are conserved between bacteria and eucaryotic cells has shown that the complex I subunit homologous to human NDUFS8 and bovine TYKY is encoded by the *Y. lipolytica NUIM* gene and the complex I subunit homologous to human NDUFS7 and bovine PSST is encoded by the *Y. lipolytica NUKM* gene (Djafarzadeh Andabili et al., submitted).

Alignments of the proteins homologous to bovine PSST (encoded by the *NUKM* genes of *Y. lipolytica* and *Neurospora crassa* and human *NDUFS7*) and of the proteins homologous to bovine TYKY (encoded by the *NUIM* genes of *Y. lipolytica* and *N. crassa* and human *NDUFS8*) demonstrate that all proteins are extremely well conserved among ascomycetous fungi and mammals in the regions affected by the Leigh mutations (cf. Fig. 1). V119 in the NUKM protein of *Y. lipolytica* corresponds to V122 in NDUFS7. It is part of a short, highly conserved stretch of hydrophobic amino acids that is predicted form a β-sheet by the method of Chou and Fasman [22]. It is separated by 32 and 30 amino acid residues, respectively, from conserved cysteines that could serve as ligands for iron-sulfur cluster N2. This part of the protein is also well conserved in procaryotes like *Paracoccus denitrificans* and *Escherichia coli*.

P98 and R121 in the NUKM protein of *Y. lipolytica* correspond to P79 and R102 in NDUFS8. These residues are immediately upstream from the first out of two CXXCXXCXXXCP motifs, that may coordinate two tetranuclear iron-sulfur clusters of the ferrodoxin-type (cf. Fig. 1).

P98 is not conserved in *E. coli*, but proline residues are found immediately adjacent to this position in *P. denitrificans*, *Rhodobacter capsulatus* and *Thermus aquaticus*. It is striking that although overall sequence homology in this region is low between eu-

**NUKM (NDUFS7/ PSST)**

```
                                                          V119
                                                           ↓
H. sapiens         FGLACCAVEM MHMAAPRYDM DRFG-VVFRA SPRQSDIMIV AGTLTNKMAP ALRKVYDQMP EPRYVVSMGSC ANGGG
Y. lipolytica      FGLACCAVEM MHVSAPRYDQ DRLG-IIFRA SPRQSDIMIV AGTLTNKMAP VLRQVYDQMP EPRWVISMGSC ANGGG
N. crassa          FGLACCAVEM MHLSTPRYDQ DRLG-IIFRA SPRQSDIMIV AGTLTNKMAP ALRQVYDQMP DPRWVISMGSC ANGGG
P. denitrificans   FGLACCAVEM MQTSMPRYDL ERFG-TAPRA SPRQSDLMIV AGTLTNKMAP ALRKVYDQMP EPRYVISMGSC ANGGG
E. coli            FGLSCCYVEM VTLFTAVHDV ARFGAEVLRA SPRQADLMVV AGTCFTKMAP VIQRLYDQML EPKWVISMGAC ANSGG
                   *** ** ***         *   * *    ** **** * * * ***   ****       ****   *  * *** * ** **
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**NUIM (NDUFS8/ TYKY)**

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                             P98                    R121
                              ↓                      ↓
H. sapiens         RGLGMTLSYL FREPATINYP FEKGPLSPRF RGEHALRRYP SGEERCIACK LCEAVCPAQA
Y. lipolytica      RGLYVVLEQF FRAPYTIIYP FEKGPVSPRF RGEHALRRYP SGEERCIACK LCEAICPALA
N. crassa          RGMYVAMEQF FRPPYTIIYP FEKGPISPRF RGEHALRRYP SGEERCIACK LCEAVCPAQA
P. denitrificans   KGFGLGMRYF VSPKPTLNYP HEKGPLSPRF RGEHALRRYP NGEERCIACK LCEAVCPAQA
E. coli            RSIWMIGLHA FAKRETRMYP EEPVYLPPRY RGRIVLTRDP DGEERCVACN LCAVACPVGC
                   *                *  **  *     **   **   * * *  ***** **  **   **
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Fig. 1. Positions of Leigh mutations in the PSST and TYKY homologues of *Y. lipolytica*. The protein sequences from various organisms were aligned using the CLUSTAL programme of the HUSAR 4.0 package (DKFZ Heidelberg, Germany). Identical amino acids are marked by asterisks. Residues forming the first half of the ferredoxin-type binding motif in TYKY and conserved cysteines in PSST are shaded in gray. The positions of the Leigh mutations are marked with arrows and the position of the residue in the *Y. lipolytica* protein is indicated.

caryotes and procaryotes, four prolines have been conserved during evolution in identical positions. This may indicate an important function for these residues in determining the three-dimensional structure of the TYKY subunit. R121 lies at the beginning of an evolutionary well conserved region which encompasses the first CXXCXXCXXXCP motif. It is the first out of two consecutive arginine residues found in all TYKY homologues, again with the exception of *E. coli*, where it is replaced by threonine and only the following arginine is conserved. Some degree of sequence deviation between complex I subunits from *E. coli* and from other procaryotes is commonly observed. This may partly be due to functional specification, since complex I from *E. coli* can use both ubiquinone and menaquinone as substrates and, as has been observed recently, translocates sodium ions instead of or in addition to protons [23].

Both the *Y. lipolytica NUIM* and *NUKM* genes were deleted in diploid cells by homologous recombination with a *LEU2* marked deletion allele. The deletion strategy for the *NUIM* gene is shown in Fig. 2. Leigh syndrome point mutations were created by site-directed mutagenesis, cloned into the single-copy replicative plasmid pINA443 and haploids carrying the deletion allele and the plasmid-borne mutant copy were isolated by sporulation and random spore analysis. The growth characteristics of all three mutants were comparable to haploid wild type strains, indicating that the complex I mutations did not markedly affect cell viability. Blue-native polyacrylamide gel electrophoresis [24] of isolated mitochondrial membranes demonstrated that the mutant versions of complex I were fully assembled and that the expression level of complex I in the mutants was comparable to wild type (data not shown).

For the determination of Michaelis-Menten parameters $V_{max}$ and $K_m$ for DBQ, complex I activity of mitochondrial membranes was assayed as electron transfer from dNADH to DBQ (cf. Fig. 3). Catalytic rates were normalised according to their NADH:HAR activity, which depends only on the presence of a functional 51 kDa subunit and can be used as a measure for total complex I content in different

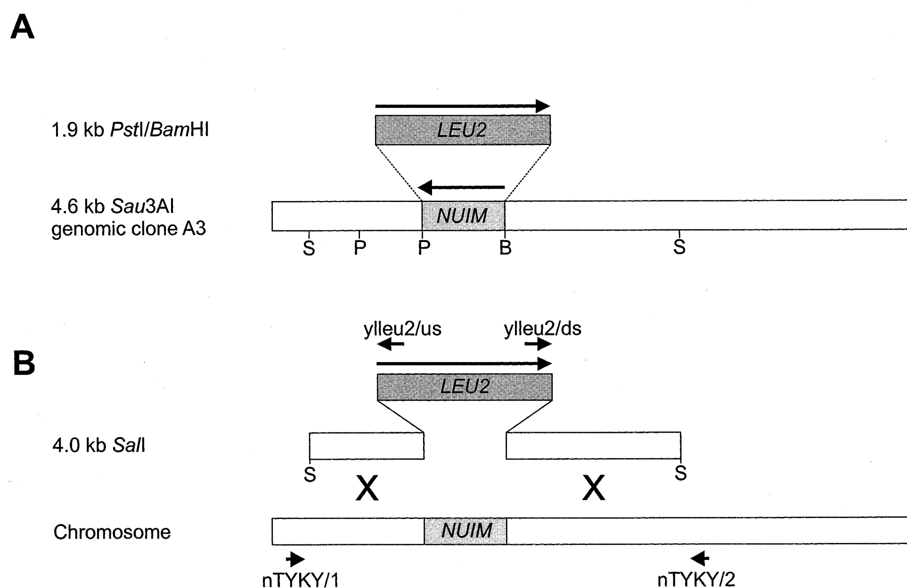


Fig. 2. Construction of the *NUIM::LEU2* deletion allele (A) and deletion of the genomic copy by homologous recombination (B). The *NUIM* open reading frame is shaded in dark gray. PCR primer pairs (ylleu2/us, nTYKY/1) and (ylleu2/ds, nTYKY/2) were used to screen *LEU2* colonies for marker insertion by homologous recombination. Restriction sites: B, *Bam*HI; P, *Pst*I; S, *Sal*I.

batches of mitochondrial membranes. In all three mutants, $V_{max}$ was found to be significantly reduced by about 50%. The $K_m$ for DBQ was unaffected in the *NUIM* R121H mutant, while it was reduced from around 20 μM to 12 μM in the NUKM V119M (PSST) and the NUIM P98L (TYKY) mutants. These two mutants also exhibited a tendency to higher $I_{50}$ values for the class B inhibitor rotenone. As indicated by a somewhat lowered $I_{50}$, the NUIM R121H and the NUKM V119M mutants exhibited a slight hypersensitivity to the type A inhibitor DQA. Type C inhibitors were not tested, as the activity of *Y. lipolytica* complex I has shown to be completely insensitive towards these compounds [19]. All data are summarised in Table 1.

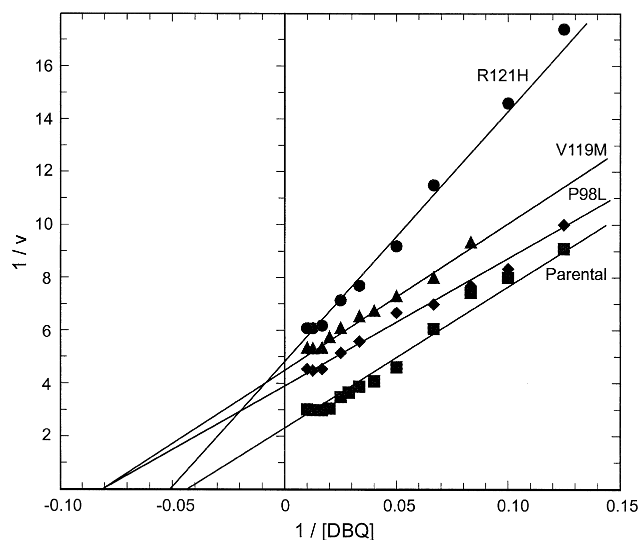


Fig. 3. Kinetic characterization of Leigh mutants in NUIM and NUKM. $K_m$ and $V_{max}$ values were determined by direct fit of the data to the standard Michaelis-Menten equation. These parameters (cf. Table 1) were used to construct the shown double-reciprocal plots of the parental strain and the three Leigh mutants. ■, parental strain; ▲, mutant NUKM V119M; ◆, mutant NUIM P98L; ●, mutant NUIM R121H.

## 4. Discussion

Point mutations within mitochondrially coded complex I subunits, such as ND4/11778, ND1/3460 or ND6/14484, have been identified in patients affected with Lebers hereditary optic neuropathy (LHON) [25]. In contrast to the mutation in ND4, there is a considerable decrease in complex I activity associated with the mutations in ND1 and ND6 [26]. The analysis of mutation ND1/3460, resulting in an A52T substitution [27,28], and of mutations of strictly conserved residues in its vicinity using the bacterium *P. denitrificans* [29] has demonstrated that this region plays an important role in ubiqui-

none reduction by complex I. The A52T substitution displayed an almost twofold increase in the $K_m$ for DBQ, but $V_{max}$ was only reduced by about 10%. Other mtDNA point mutations, such as ND4/11696 and ND6/14459, cause LHON with additional dystonia, accompanied by lesions in the caudate and putamen visible on MRI [30,31]. While the unravelling of these mtDNA mutations has firmly established the functional role of mitochondrially encoded subunits in the interaction of complex I with the substrate ubiquinone, recent findings have underlined the importance of nuclear coded subunits in ubiquinone binding and reduction as well as in the proton translocating function of the enzyme. The 49 kDa homologue is the target of a piericidin A resistant mutation in *R. capsulatus* [32]. Bovine PSST can be labelled with a photoreactive derivative of pyridaben [33] and point mutations in *NUKM*, the homologue of this subunit in *Y. lipolytica*, lead to resistance to DQA and hypersensitivity to rotenone [19]. Iron-sulfur cluster N2, which stands out by the striking pH dependence of its midpoint potential [34], is generally assumed to act as the direct reductant for ubiquinone and has been proposed to be directly involved in proton translocation [35,36]. Based on mutagenesis studies in *E. coli* [37] and *R. capsulatus* [38], both PSST and TYKY, respectively, have been proposed to carry iron-sulfur cluster N2. The recent finding that conserved acidic residues in the PSST homologue of *Y. lipolytica* interact with cluster N2 and may contribute to a network of hydrogen bonds that channels protons to or away from cluster N2 [19] supports the view that this subunit is involved in proton translocation by complex I. It is remarkable that all these functionally important subunits, namely the 49 kDa, PSST, TYKY and ND1, together with ND5 are conserved between complex I and membrane-bound Ni-Fe hydrogenases from archebacteria [39]. These subunits may be regarded as the catalytic core of the enzyme and appear as prime candidates for deleterious mutations affecting its catalytic function.

In the present work we have analysed the effect of three mutations identified in patients affected with Leigh syndrome that are located in two subunits of this catalytic core of complex I, namely PSST and TYKY. The point mutations involved residues that are fully conserved among eucaryotes and were reconstructed in the obligate aerobic yeast *Y. lipolytica*, a new model system which allows the application of the powerful methods of yeast genetics to the analysis of mitochondrial complex I. This approach may not only help to understand the aetiology of Leigh syndrome, which could involve reduced electron transport and proton pumping efficiency or the generation of harmful radical oxygen species, but should also provide useful hints on catalytically important regions within complex I.

We have therefore examined the in vitro kinetics of the Leigh mutant versions of complex I from *Y. lipolytica*, in order to detect alterations in the interaction between complex I and the substrate ubiquinone or its inhibitory analogues. We found that all three mutations affected electron transport (Table 1, Fig. 3): $V_{max}$ of complex I in mitochondrial membranes was reduced by about 50% in all three mutant strains. Among the two point mutations found in NDUFS8/NUIM, the R121H missense mutation resulted in a lower $V_{max}$ than the P98L mutation. Remarkably, for two mutants, NUKM V119M and NUIM P98L, the $K_m$ for ubiquinone was decreased by about a factor of two to 12 μM, resulting in an unchanged specificity constant as compared to the parental strain. All three mutations also seemed to

Table 1

| Strain | DBQ | | | $I_{50}$ | |
|---|---|---|---|---|---|
| | Normalized $V_{max}$ (μmol $min^{-1}$ $mg^{-1}$) | % | $K_m$ (μM) | DQA (nM) | Rotenone (μM) |
| Parental | 0.44 ± 0.02 | 100 | 22 ± 2 | 20 | 0.5 |
| NUKM V119M | 0.22 ± 0.01 | 50 | 12 ± 1 | 10 | 0.7 |
| NUIM P98L | 0.25 ± 0.01 | 57 | 12 ± 1 | 20 | 0.7 |
| NUIM R121H | 0.20 ± 0.01 | 45 | 19 ± 2 | 10 | 0.5 |

affect the inhibitory potency of DQA and/or rotenone, but these effects were not very pronounced (cf. Table 1). Thus, the reconstruction of the human Leigh mutations in the yeast *Y. lipolytica* indeed resulted in catalytically impaired complex I. In contrast to the LHON mutation ND1/3460 that was reconstructed in *P. denitrificans* [29], a substantial reduction of $V_{max}$, but an unchanged or even lowered $K_m$ for DBQ, was observed in all three mutants studied here. When using a microorganism as a model, it is inherently impossible to find out how a given functional defect at the level of complex I translates into characteristic organ and tissue specific patterns of malfunction that is then diagnosed as a certain human disorder. However, the observation that the mutations in NUKM and NUIM had so similar effects on $V_{max}$ of complex I and cause Leigh syndrome, but that mutation ND1/3460 increases the $K_m$ for DBQ but leaves $V_{max}$ almost unchanged [29] and causes LHON may provide a first clue towards a molecular understanding of the aetiology of human disorders caused by complex I defects.

With regard to the structure/function relationships in complex I our results provide yet another indication that some of the nuclear coded subunits are directly involved in quinone chemistry and may even be part of the quinone reactive site of complex I as suggested previously [32]. It seems sensible to assume that the quinone head group of ubiquinone must be bound to the hydrophilic part of complex I, in order to come close to its direct reductant, cluster N2. The quinone tail may still interact mainly with the membrane part of the enzyme, e.g. with the ND1 subunit, mutations which are known to reduce the affinity of complex I for ubiquinone, and the ND5 subunit, which is also among the core subunits conserved between membrane bound Ni-Fe hydrogenases from archaea and complex I.

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