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# Tight binding of NADPH to the 39-kDa subunit of complex I is not required for catalytic activity but stabilizes the multiprotein complex

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**Abstract**

In addition to the 14 central subunits, respiratory chain complex I from the aerobic yeast *Yarrowia lipolytica* contains at least 24 accessory subunits, most of which are poorly characterized. Here we investigated the role of the accessory 39-kDa subunit which belongs to the heterogeneous short-chain dehydrogenase/reductase (SDR) enzyme family and contains non-covalently bound NADPH. Deleting the chromosomal copy of the gene that codes for the 39-kDa subunit drastically impaired complex I assembly in *Y. lipolytica*. We introduced several site-directed mutations into the nucleotide binding motif that severely reduced NADPH binding. This effect was most pronounced when the arginine at the end of the second β-strand of the NADPH binding Rossman fold was replaced by leucine or aspartate. Mutations affecting nucleotide binding had only minor or moderate effects on specific catalytic activity in mitochondrial membranes but clearly destabilized complex I. One mutant exhibited a temperature sensitive phenotype and significant amounts of three different subcomplexes were observed even at more permissive temperature. We concluded that the 39-kDa subunit of *Y. lipolytica* plays a critical role in complex I assembly and stability and that the bound NADPH serves to stabilize the subunit and complex I as a whole rather than serving a catalytic function.


*Keywords:* Mitochondria; Complex I; NADH:ubiquinone oxidoreductase; 39-kDa subunit; Short-chain dehydrogenases/reductases; NADPH; *Yarrowia lipolytica*

## 1. Introduction

NADH:ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport chain catalyses the transfer of two electrons from NADH to ubiquinone, coupled to the translocation of four protons across the membrane [1]. As revealed by electron microscopic studies the enzyme has an L-shaped structure with two arms: a membrane-embedded part and a part protruding into the matrix called the peripheral arm [2]. While the proton pump is located in the hydrophobic part, the hydrophilic part exhibits NADH dehydrogenase activity and is also responsible for ubiquinone reduction [3]. Mitochondrial complex I has a higher complexity and molecular mass than the bacterial enzyme. In most bacteria complex I is composed of only 14 subunits. They represent the minimal form of the enzyme required to ensure the coupling of electron transfer with proton pumping. All of them are conserved in eucaryotic complex I [4]. In addition, complex I from eucaryotic organisms contains a large number of "accessory" subunits: 32 subunits in bovine heart mitochondrial complex I [5], 23–25 subunits in the fungal enzyme [6,7] and about 16–28 subunits in photosynthetic organisms like *Vicia faba* [8], *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* [9] were identified. Only some accessory subunits have been analysed in detail and the function of most of them remains unknown, although involvement in enzyme assembly and stability or additional functions unrelated to NADH:ubiquinone oxidoreductase activity have been discussed [1].

*Abbreviations:* BN-PAGE, blue-native polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DBQ, n-decylubiquinone; dNADH, deamino-nicotinamide-adenine-dinucleotide (reduced form); dSDS-PAGE, doubled sodium-dodecyl sulfate polyacrylamide gel electrophoresis; FMN, flavine-mononucleotide; HAR, hexa-ammine-ruthenium(III)-chloride; NBT, nitro blue tetrazolium; RP-HPLC, reverse phase high-pressure liquid chromatography; SDS, sodium-dodecyl sulfate; SDR, short-chain dehydrogenase/reductase (enzyme family); YPD, yeast peptone dextrose (complete medium)

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The nuclear-encoded, accessory 39-kDa subunit is related to the SDR (short-chain dehydrogenases/reductases) enzyme family [10]. Members of this family function in different redox and isomerization reactions and contain a conserved NAD(P)H-binding site near their N-termini. It was suggested that the 39-kDa subunit may be involved in intramitochondrial fatty acid synthesis [11].

Here, we have deleted the gene encoding the 39-kDa (NUEM) subunit of complex I in the aerobic yeast *Yarrowia lipolytica* and have introduced several point mutations in the putative nucleotide binding site of this subunit. Our results indicate that the 39-kDa subunit plays an important role for complex I assembly and stability in *Y. lipolytica*, but that NADPH binding is not critical for the formation of catalytically active complex I.

## 2. Materials and methods

### 2.1. Deletion of the NUEM gene

The *NUEM* gene encoding the 39-kDa subunit of *Y. lipolytica* complex I was deleted by homologous recombination with a *URA3*-marked deletion allele. First, a genomic 4.91 kb PCR product generated using primers TGTGCGGCCGCGTTCGACATCTAACTGGCA and AGAGTCGACGTCTCAGGAATCCTTCA was cloned into the pBluescript SK(−) vector (Stratagene) using *Not*I/*Sal*I. A region of about 1.2 kb including the complete *NUEM* open reading frame was replaced by a *Bam*HI/*Eco*RI restricted PCR product carrying the *URA3* gene from *Y. lipolytica* in opposite orientation of the original *NUEM* gene. The 5.2 kb *nuem*::*URA3* *Not*I/*Sal*I fragment was used for transformation of haploid *Y. lipolytica* GB10 cells (*3*0 *Htg2 Mat*B *ndh2i ura3*-302 *leu2*-270 *lys*-1). Transformants were selected on minimal media without uracil and screened for homologous recombination at the chromosomal *NUEM* locus by PCR and Southern blot analysis.

### 2.2. Generation of point mutations

To produce the "parental strain" used in this study, the genomic *NUEM* PCR product was excised from pCR 2.1 (Invitrogen) using *Eco*RI, cloned into the "shuttle"-vector pUB4 harboring the *HygB*$^R$ gene and transformed into the *nuem*Δ strain. Plasmid pUB4/*NUEM* was also used as template for site directed mutagenesis according to the QuikChange™ (Stratagene) protocol. To check for the presence of the desired mutation and the absence of inadvertent sequence changes, the complete open reading frames of the mutagenised gene copies were sequenced using an ABI Prism 310 genetic analyser (Applied Biosystems).

### 2.3. Purification of complex I

*Yarrowia lipolytica* strains were grown in YPD medium at 28 °C in a 10 l fermenter (Biostat E; Braun, Melsungen). Fermentation lasted 14–18 h. Complex I was purified from isolated mitochondrial membranes, which were solubilised with *n*-dodecyl-β-D-maltoside as described [12] with minor modifications. Purification was achieved by $Ni^{2+}$-affinity chromatography with a modest reduction of the imidazole concentration from 60 mM to 55 mM in the equilibration and washing buffer and subsequent gel filtration using a TSK4000 column.

### 2.4. Native gel electrophoresis

Blue-native polyacrylamide gel electrophoresis [13] was used to separate the components of the mitochondrial respiratory chain in mitochondrial membranes of *Y. lipolytica*, which were prepared according to standard procedures [14]. 500 μg of total protein was solubilised with 1.0 g/g *n*-dodecyl-β-D-maltoside or 3.0 g/g digitonin and 500 mM amino caproic acid. The resulting solubilised mitochondrial membranes were put on 4–13% gradient gels. Individual lanes were cut out, incubated for 30 min in 1% SDS and applied on 10 or 16% Tricine SDS gels, for resolution in the second dimension. Gels were stained with Coomassie blue G 250. It should be noted that even under mild solubilisation conditions using digitonin rather that laurylmaltoside no supercomplexes are observed by BN-PAGE of *Y. lipolytica* membranes. For in-gel detection of the NADH dehydrogenase activity of complex I, unfixed BN-PAGE gels were incubated for 5 min in a solution containing 3 mM nitro blue tetrazolium (NBT) and 120 μM NADH. To stop the reaction, gels were incubated in 50% methanol and 10% acetic acid.

### 2.5. Doubled SDS-polyacrylamide gel electrophoresis

Tricine dSDS-PAGE was used to separate the subunits of complex I from *Y. lipolytica* and was performed as described [15]. Briefly, lanes from 1D-gels (10% polyacrylamide, 6 M urea) were incubated in acidic solution containing 100 mM Tris, 150 mM HCl, pH 2 for 30 min and analysed by SDS-PAGE as a second dimension using 16% polyacrylamide. The 2D-gels were stained with Coomassie blue G 250.

### 2.6. Western blot

To investigate protein expression, a semidry immunoblotting procedure was applied using a polyvinylidendifluoride membrane (Immobilon™ P, Millipore). Membranes and gel-blotting paper were incubated in blotting-buffer (cathode buffer: 300 mM 6-aminocaproic acid, 30 mM Tris, pH 9.2; anode buffer: 300 mM Tris, 100 mM Tricine, pH 8.7). 10% gels were blotted for 2 h at 200 mA and 20 V. Membranes were stained with Coomassie blue G 250, and the positions of complexes I, III, IV and V were recorded. After destaining with methanol, membranes were incubated for 30 min in PBS buffer containing 0.4% (w/v) Tween-20 to reduce unspecific binding. After washing in PBS buffer (containing 0.1% (w/v) Tween-20) once for 15 min and twice for 5 min, membranes were incubated over night with primary monolyclonal antibodies (undiluted hybridoma cell culture supernatant) against the 51-kDa, 49-kDa, 39-kDa or NESM subunits of complex I. After washing once for 15 min and twice for 5 min, incubation for 1 h with anti-mouse IgG peroxidase conjugate (diluted 1:10.000 in PBS) was performed. Proteins were detected by enhanced chemiluminescence (ECL).

### 2.7. Catalytic activity of complex I

Activity measurements of complex I were done according to previously published protocols [16] using a Shimadzu MultiSpec-1501 or a Molecular Devices SPECTRAmax PLUS$^{384}$ spectrophotometer by following dNADH oxidation at 340 minus 400 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). Since all strains contained the internal version of alternative dehydrogenase (NDH2i) [17], which is able to oxidize NADH, but not dNADH (deamino-NADH), for measurements of complex I activity dNADH was used to discriminate between complex I and the alternative enzyme. Complex I content was estimated as detergent- and inhibitor-insensitive dNADH:HAR activity. The reaction was started by the addition of 25 μg (total protein) or 3 μg of mitochondrial membranes or isolated complex I, respectively. For measurement of complex I activity, dNADH:DBQ activity was measured at 60 μM DBQ and 100 μM dNADH. The reaction was started by adding final concentrations of 30–50 μg protein/ml mitochondrial membranes or of 1 μg/ml isolated enzyme. Purified enzyme samples were reactivated with asolectin at a 1:1 (w/w) protein-to-lipid ratio for measurement of complex I activity [18].

### 2.8. EPR-spectra

Low temperature EPR spectra were obtained with a Bruker ESP 300E spectrometer equipped with a liquid helium continuous flow cryostat, ESR 900 from Oxford Instruments. Samples were mixed with NADH in the EPR tube and frozen in liquid nitrogen after 30 s reaction time. Spectra were recorded at 12 K with the following instrument settings: microwave frequency 9.475 GHz, microwave power 1 mW, modulation amplitude 0.64 mT. Under these conditions spectra show contributions from iron–sulfur clusters N1, N2, N3 and N4.

### 2.9. Extraction of NADPH and FMN from mitochondrial membranes and complex I

One volume of mitochondrial membranes at 10 mg/ml protein was mixed with one volume of 1% (w/v) potassium cholate in Tris–acetate buffer (0.15 M KCl in 10 mM Tris–acetate, pH 7.5) and incubated for 30 min on ice. After centrifugation at 35,000×*g* for 1 h the membranes were washed twice in washing buffer containing 1 mM EDTA, 0.1 mM PMSF in 10 mM Tris–acetate (pH 7.5). The pellet was resuspended in washing buffer. Extraction of non-covalently bound nucleotides was performed as described in [19] with some modifications. 3 mg of protein were lyophilized and then extracted for 10 min with 500 μl of chloroform/methanol (2:1, v:v) by vigorous shaking on ice. After addition of 600 μl of 0.1 M NaOH adjusted to pH 10 with glycine the sample was incubated for 15 min with vigorous shaking on ice. After centrifugation for 10 min at 11,000×*g* the upper phase was filtered through a cellulose minifilter (Minisart-RC/SRP, 0.20 μM, Sartorius), lyophilized and then redissolved in 50 μl of filtrated water. The same extraction procedure was carried out with complex I and standard solutions containing 1–5 nmol of nucleotides (NADH, NADPH, FAD or FMN) and 1 mg/ml of bovine serum albumin. 1 mg of purified complex I was extracted with 200 μl chloroform/methanol and incubated with 300 μl 0.1 M NaOH.

### 2.10. Determination of NADPH and FMN

HPLC was performed using a Hibar RT 250-4, LiChrospher 100, RP 18 (5 μm) reversed-phase column with a pre-column containing the same material (Merck, Germany), combined with a L-6210 Intelligent Inert Pump (Merck/Hitachi) and with a L-400/L-4200 UV/Vis-detector (Merck/Hitachi) set to 340 nm. Elution occurred over 75 min at a flow rate of 0.4 ml/min with 1% (v/v) methanol in 0.05 M potassium phosphate (pH 6), followed by a linear gradient from 1% to 50% (v/v) methanol in phosphate buffer (pH 6) over 40 min. Then a methanol/buffer (1:1 v/v) step of 5 min was followed by a linear gradient from 50% to 100% methanol over 15 min. Finally, the column was flushed with 100% methanol for 10 min. Quantitative determination of nucleotides in complex I was carried out with standard solutions of nucleotides (1–5 nmol) that were mixed with BSA and treated in the same way as the complex I samples.

## 3. Results

### 3.1. Absence of the 39-kDa subunit drastically impairs complex I assembly in Y. lipolytica

We isolated mitochondrial membranes from a strain lacking the NUEM gene encoding the 39-kDa subunit of *Y. lipolytica* complex I and checked for complex I assembly by two dimensional BN/Tricine SDS-PAGE. Using this method, complex I was identified in membranes of the parental strain, but could not be detected in the *nuem*Δ strain, even if 0.7 g rather than the standard amount of 1.0 g of lauryl-maltoside per g protein were used for solubilisation (data not shown).

Inhibitor sensitive dNADH: ubiquinone oxidoreductase activity is only catalyzed by fully assembled and functional complex I. In contrast, dNADH:HAR oxidoreductase activity monitors a partial reaction of complex I that is merely catalyzed by its FMN containing 51-kDa subunit. Assaying this reaction is useful to estimate the total content of complex I and possible subcomplexes containing the 51-kDa subunit. Mitochondrial membranes from strain *nuem*Δ exhibited no dNADH:ubiquinone oxidoreductase activity, but as compared to the parental strain, a dNADH:HAR activity of 16% was still detectable (Table 1). Because this residual dNADH:HAR activity could have resulted from subcomplexes containing the NUBM (51-kDa) subunit, two-dimensional gels (BN/SDS-PAGE) of parental and *nuem*Δ membranes were subjected to Western blotting with monoclonal antibodies against the 51-kDa (NUBM), 49-kDa (NUCM), the 39-kDa and NESM (homologue of bovine ESSS) subunits. As shown in Fig. 1, even with this sensitive method, only a small amount of assembled complex I was detectable in the deletion strain (Note that the amount of membranes used in the Western blot analysis was doubled for the deletion strain). Although this assembled complex lacked the deleted 39-kDa subunit, its migration behavior in the first dimension (BN-PAGE) was essentially unchanged. This suggested that most, if not all other subunits of complex I were still present. In contrast to the parental strain, complex I subunits were also detected in three different subcomplexes and at the front of the native gel, where they most likely migrated as individual proteins (Fig. 1). From their migration behavior relative to other multiprotein complexes of the respiratory chain, it could be estimated that the masses of the three subcomplexes was around 700, 400 and 200 kDa (marked 1–3 in Fig. 1). It was beyond the scope of this study to further analyse their nature and subunit composition. Overall, the total amount of immunodetectable complex I subunits was much smaller in the deletion strain than in the parental strain. These results show that the 39-kDa subunit plays a critical, although not essential role for the formation of stable complex I in *Y. lipolytica*.

### 3.2. Mutations in the NADPH binding site destabilize complex I

The NADPH binding site in the 39-kDa subunit consists of a βαβ Rossman fold (Fig. 2). This structural motif is found in many NAD(P)H binding sites and is characterized by three highly conserved glycine residues, spaced GXXGX(X)G. They are located at the junction between the first β-strand and the connecting α-helix. The middle glycine makes close contact to the pyrophosphate moiety of the cofactor and is crucial for nucleotide binding. In NADPH binding sites, a basic residue at the end of the second β-strand interacts with the additional

Table 1
Activities of mitochondrial membranes and isolated complex I

| Strain | Mitochondrial membranes | | | Isolated complex I | |
|---|---|---|---|---|---|
| | HAR activity[a] % | Ubiquinone reductase activity[b] | | Ubiquinone reductase activity[b] | |
| | | μmol $min^{-1}$ $mg^{-1}$ | % | μmol $min^{-1}$ $mg^{-1}$ | % |
| Parental | 100 | 0.41±0.06 | 100 | 3.1±0.2 | 100 |
| *nuem*Δ | 16 | n.d.[c] | – | n.d. | – |
| G43A | 50 | 0.37±0.03 | 90 | 2.4±0.2 | 78 |
| G43V | 29/43[d] | n.d. | – | n.d. | – |
| R65D | 64 | 0.33±0.01 | 80 | 0.4±0.07 | 13 |
| R65L | 50 | 0.39±0.04 | 95 | 1.0±0.08 | 32 |

[a] Specific dNADH:HAR oxidoreductase activity (100%=1.4 μmol $min^{-1}$ $mg^{-1}$); corresponds to the content of complex I and subcomplexes containing the 51-kDa subunit.
[b] dNADH:DBQ oxidoreductase activity normalized for dNADH:HAR activity of parental sample.
[c] n.d. — not detectable.
[d] In cells grown at 28 °C and 20 °C, respectively.

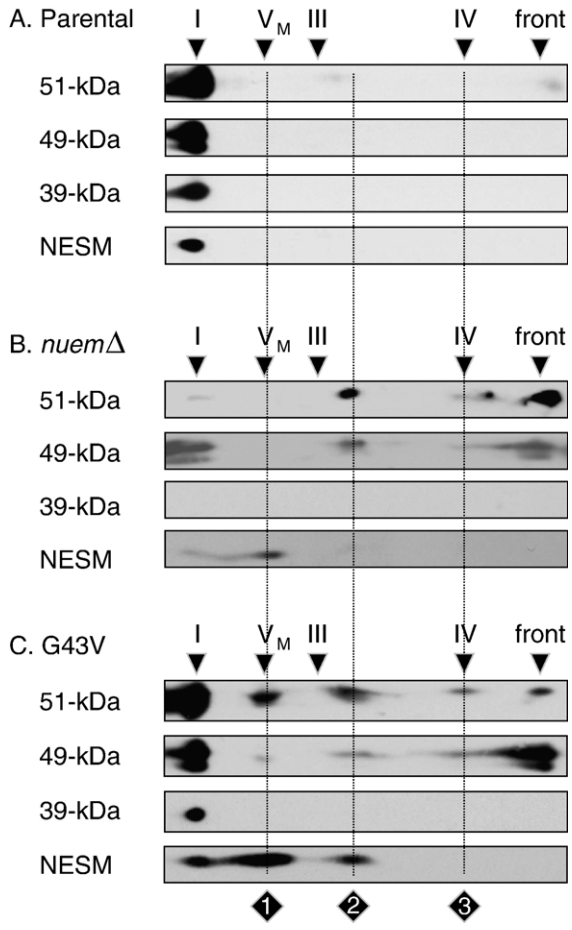


Fig. 1. Western blot analysis of mitochondrial membranes prepared from the parental, the NUEM deletion (*nuem*Δ) and the G43V mutant strains. Mutant G43V was grown at 20 °C. Respiratory chain complexes were separated by 2 D (BN/SDS)-PAGE following solubilisation with 1.0 g/g lauryl-maltoside. Mouse monoclonal antibodies directed against the 51-kDa (NUBM), 49-kDa (NUCM) and 39-kDa (NUEM) and NESM subunits of *Y. lipolytica* complex I were used. Fully assembled complex I (I; ~900 kDa) and the positions of complex III dimer (III; ~450 kDa), complex IV monomer (IV; ~200 kDa) and complex V monomer (V; ~700 kDa) were identified by Coomassie blue staining of the PVDF membranes, prior to immunodetection and are indicated by triangles. Three subcomplexes (1, 2, 3) are indicated by dotted lines, labelled with diamonds. Signals at the front of the gel most likely represent "free" subunits. A, 0.5 mg mitochondrial membranes from parental strain in 1.0 g/g laurylmaltoside; B, 1.0 mg mitochondrial membranes from *nuem*Δ strain in 1.0 g/g laurylmaltoside, C, 0.5 mg mitochondrial membranes from mutant G43V grown at 20 °C in 3.0 g/g digitonin.

phosphate group and is therefore responsible for selective binding of this particular nucleotide [20]. In the present study, we focused on these two critical residues within the 39-kDa subunit. Glycine-43 was changed to alanine and valine, while arginine-65 was changed to leucine and aspartic acid. As judged by BN-PAGE, mitochondrial membranes from mutants G43A, R65L and R65D contained fully assembled complex I, which exhibited in-gel NADH dehydrogenase activity (data not shown). dNADH:HAR oxidoreductase activity, was decreased to 50–64% (Table 1), but no significant changes in growth rates were observed (data not shown). The normalized specific ubiquinone reductase activity was found to reach near wild type levels in all three mutants (Table 1).

The dNADH:HAR oxidoreductase activity of mitochondrial membranes from mutant G43V suggested a content of 29% of fully or partially assembled complex I in the parental strain. However, no complete complex I was detectable in this mutant by BN-PAGE (data not shown). Based on the assumption that the change of glycine-43 to valine may have led to formation of a more labile enzyme, mutant G43V was also grown at lower temperature. In fact, in mitochondrial membranes from the G43V strain grown at 20 °C rather than 28 °C, dNADH:HAR oxidoreductase activity was increased to 43% of the parental strain activity. Respiratory chain complexes of mutant G43V grown under these more permissive conditions were separated by BN-PAGE under mild conditions in the presence of 3 g digitonin/g protein [13]. While still no assembled complex I could be detected by Coomassie staining, Western blot analysis using antibodies against the NUBM (51-kDa), NUCM (49-kDa), NUEM (39-kDa) and NESM subunits revealed the presence of some fully assembled complex I (Fig. 1). Similar as in the deletion strain, three subcomplexes and some "free" subunits at the front of the gel could be found. Due to the much lower amounts of subcomplexes in the deletion strain, some Western blot signals (i.e. for the 51-kDa and 49-kDa subunits in subcomplex 1 and the NESM subunit in subcomplex 2) may have escaped detection (Fig. 1). This leaves some uncertainty about the identity and exact composition of these subcomplexes and further studies will be needed to resolve this issue.

At any rate, the total amount of subcomplexes containing the 51-kDa subunit was clearly much higher in G43V membranes than in the deletion strain, which is consistent with the substantially elevated dNADH:HAR oxidoreductase activity of membranes from the point mutant (Table 1). However, still no dNADH:ubiquinone oxidoreductase activity was detectable (Table 1). Taken together these results indicated that mutation G43V caused significant destabilization and/or incomplete assembly of complex I.

From mutants G43A, R65L and R65D, assembled complex I could be purified by standard procedures. As judged from dSDS-PAGE, all subunits were retained in the mutant enzymes in amounts comparable to parental complex I, with the sole exception of the sulfurtransferase protein. This subunit, which was only recently identified in *Y. lipolytica* complex I by mass spectrometry [21], was present in reduced amounts in complex I from mutant R65L. In mutant R65D, only traces of sulfurtransferase were detectable (not shown).

EPR spectra of iron sulfur clusters N1, N2, N3 and N4 of complex I purified from mutants G43A, R65L and R65D


Fig. 2. Sequence of the NADPH binding site in the 39-kDa subunit of *Y. lipolytica* complex I. Conserved glycines and the conserved basic residue at the end of the second β-strand are shaded in grey. The amino acid residues, which were altered by site-directed mutagenesis, are marked with arrows. Helical (H) and extended (E) regions were predicted using the PROF algorithm [25].

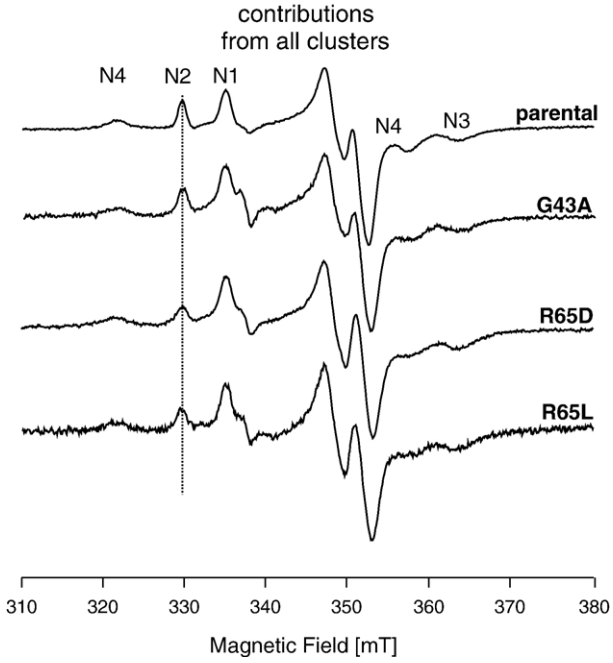


Fig. 3. EPR spectra of complex I purified from the parental and the G43A, R65D and R65L mutant strains. Complex I was reduced with NADH and spectra were recorded at a sample temperature of 12 K. Spectra show contributions from clusters N1, N2, N3 and N4 [26]. In the R65D and R65L mutants, the signals of cluster N2 are slightly altered. The position of the $g_z$ signal of cluster N2 is indicated by a dotted line, the positions of the $g_x$ and $g_y$ signals are superimposed by signals from other clusters.

showed no significant changes compared to the parental strain, except that the spectra of mutants R65D and R65L exhibited slight alterations in the signal for iron–sulfur cluster N2 (Fig. 3). Specific dNADH:DBQ oxidoreductase activity of purified complex I, measured after relipidation with asolectin, was only slightly reduced in mutant G43A. However, complex I purified from mutants R65D and R65L was only partially reactivated by phospholipids (Table 1).

### *3.3. Mutations R65L and R65D impair binding of NADPH to the 39-kDa subunit*

In order to determine the NADPH content of isolated complex I, we extracted it with an alkaline chloroform/methanol mixture. Analysis of extracts from purified complex I of the parental strain by reversed-phase HPLC (RP-HPLC) yielded peaks for FMN and NADPH, but not for NADH (Fig. 4A). Using standard solutions containing 0.5–5 nmol of NADH, NADPH and FMN, a retention time of 31 min was determined for NADH, whereas due to its higher polarity, NADPH eluted at 27 min retention time (data not shown). A nucleotide content of 0.56 nmol of NADPH and 0.93 nmol FMN per mg of complex I was determined for the parental strain (Table 2). With a total molecular mass for complex I of about 930 kDa, this corresponded to 1 FMN per complex I, while the content of NADPH was substoichiometric. In extracts of complex I purified from mutant G43A, the same content of FMN, but a clearly reduced amount of NADPH was detected (Fig. 4A).

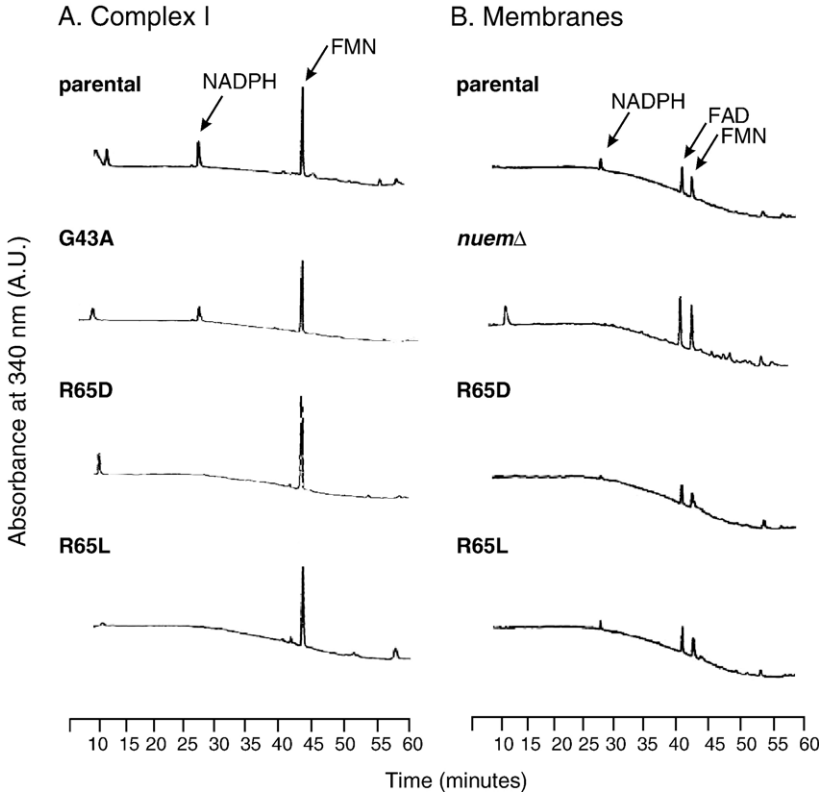


Fig. 4. RP-HPLC analysis of hydrophilic extracts. Peaks corresponding to NADPH, FAD and FMN are indicated by arrows. (A) Extracts of purified complex I from the parental strain and mutants G43A, R65D and R65L. (B) Extracts of mitochondrial membrane extracts of purified complex I from the parental strain, the *nuem*Δ strain and mutants R65D and R65L.

Table 2
Content of NADPH and FMN in complex I from parental and mutant strains

| Strain | NADPH | FMN |
|---|---|---|
| | nmol/mg of complex I | |
| Parental | 0.56 | 0.93 |
| G43A | 0.35 | 0.81 |
| R65D | n.d. | 0.99 |
| R65L | n.d. | 0.61 |

Also the FMN content of complex I from mutant R65D was unchanged, whereas it was decreased in mutant R65L to about two thirds of the parental strain value (Table 2). However, no NADPH could be extracted from purified complex I of these two mutants (Fig. 4A) indicating strongly impaired binding of this nucleotide to the 39-kDa subunit.

We then asked whether mutations R65L and R65D had completely abolished the nucleotide binding capacity of the 39-kDa subunit or whether the more weakly bound NADPH got lost upon complex I purification. To answer this question, we extracted nucleotides also from mitochondrial membranes. Before extraction, it was necessary to wash the mitochondrial membranes with cholate in order to reduce the content of soluble and weakly membrane-associated proteins and to reduce background signals. While this step had minor effects on the dNADH:ubiquinone oxidoreductase activity of membranes from the parental strain and mutant R65L, it reduced this activity in mutant R65D by about 50%. As shown in Fig. 4B an additional compound eluted at around 41 min in RP-HPLC of extracts of parental strain membranes. This peak was assigned to FAD and resulted from respiratory chain enzymes like complex II. Also FMN and NADPH were clearly detectable in membrane extracts, but the small amounts of nucleotides present prevented reliable quantification. It should be noted that a peak for FMN could be observed also with mitochondrial membranes from strain *nuem*Δ (Fig. 4B). This is in line with the Western Blot analysis, which demonstrated that some complex I, subcomplexes containing the 51-kDa subunit, and free 51-kDa subunit were present in the *nuem*Δ strain (Fig. 1 and Table 1). NADPH, on the other hand, could not be detected in membranes from strain *nuem*Δ. From this observation it follows that all the NADPH that is found in membranes prepared from parental or point mutant strains is indeed derived from complex I.

As judged from the relative amounts of FAD and FMN, mitochondrial membranes from mutants R65D and R65L contained normal amounts of FMN, whereas the NADPH content was significantly reduced (Fig. 4B). This effect was most pronounced with mutant R65D, where only traces of NADPH were found. Thus, the mutations within the nucleotide fold had markedly reduced but not completely abolished the affinity for NADPH.

## 4. Discussion

Our results show that NADPH is tightly bound to the 39-kDa subunit of *Y. lipolytica* complex I. This is in line with earlier studies with bovine [11] and *N. crassa* [19] complex I which also suggested that this nucleotide is associated with the 39-kDa subunit. Assembly and stability of *Y. lipolytica* complex I was severely impaired by deleting the *NUEM* gene encoding the 39-kDa subunit, but a small amount of complex I was still detectable in mitochondrial membranes from the deletion strain. This effect on the stability of complex I seems to be less pronounced in *N. crassa* were a 39-kDa subunit deletion strain was reported to still contain one third of assembled complex I that could be purified in an inactive form [19]. However, instability of complex I purified from the *N. crassa* deletion strain is also evident since a markedly reduced N2 signal was reported for the purified mutant enzyme [19].

To discriminate whether these global effects reflected a role of the 39-kDa subunit as a whole or only of its NADPH binding site and to obtain more insight into the function of the nucleotide binding site, we introduced point mutations at two critical positions within the nucleotide binding site of the *Y. lipolytica* protein. When we complemented the *nuem*Δ strain with plasmids coding for different versions of the mutated 39-kDa subunit, partial recovery of complex I assembly was evident in all four cases: mutant G43V exhibited a temperature sensitive assembly phenotype, while membranes from mutants G43A, R65D and R65L exhibited moderately reduced contents of complex I.

NADPH binding was markedly reduced in the latter three mutants: NADPH was completely absent from purified complex I of mutants R65D and R65L and a significantly reduced amount of this nucleotide was extracted from purified complex I of G43A. Also in mitochondrial membranes, only residual amounts of NADPH were detectable indicating that the mutations had severely reduced the affinity of the 39-kDa subunit for NADPH. Remarkably, this did not go in parallel with a reduction of the specific ubiquinone reductase activity that was essentially unchanged in mitochondrial membranes from the three mutants. Therefore, in contrast to an earlier suggestion [19], our results seem to rule out that the NADPH binding site within the 39-kDa subunit is critical for the biosynthesis of functional complex I. It also seems impossible that the 39-kDa subunit, possibly in cooperation with the complex I associated acyl carrier protein, is involved in the biosynthesis of a postulated but as yet unidentified additional lipid cofactor of complex I [22].

Our findings rather suggest that the 39-kDa subunit is of general importance for proper assembly of eukaryotic complex I and that tight binding of NADPH supports this function most likely by stabilizing the structural fold of the subunit. The fact that intact and partly active complex I could be isolated even when essentially all NADPH was lost during the purification procedure in mutants R65D and R65L (compare Tables 1 and 2), may suggest that the presence of the bound nucleotide is more important during biosynthesis than in fully assembled complex I. On the other hand, incomplete reactivation of the purified enzymes from these mutants by phospholipids suggested some structural destabilization of complex I. The reduced content of the sulfurtransferase subunit in complex I purified from the mutant strains may be taken as another

indication for some structural changes associated with the loss of bound NADPH.

Overall our results suggest that the NADPH bound to the 39-kDa subunit is primarily important for structural integrity of the subunit and thereby stabilizes complex I as a whole. Tightly bound, non-catalytic nucleotides are found in $F_1F_O$-ATPase containing three non-exchangeable ATPs [23]. Tumor supressor CC3/Tim30 is also a member of the SDR-family and contains NADPH without known catalytic function [24]. Further studies will be required to find out whether the NADPH bound to the 39 kDa may have some kind of regulatory function, an option that cannot be fully excluded at present.

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