Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1767 (2007) 393–400
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbabio

Characterization of a subcomplex of mitochondrial NADH:ubiquinone oxidoreductase (complex I) lacking the flavoprotein part of the N-module

Volker Zickermann, Klaus Zwicker, Maja A. Tocilescu, Stefan Kerscher, Ulrich Brandt *

Johann Wolfgang Goethe-Universität, Fachbereich Medizin, Molekulare Bioenergetik, Centre of Excellence Frankfurt "Macromolecular Complexes", D-60590 Frankfurt am Main, Germany

Received 7 February 2007; received in revised form 2 March 2007; accepted 5 March 2007
Available online 15 March 2007

Abstract

Mitochondrial NADH:ubiquinone oxidoreductase is the largest and most complicated proton pump of the respiratory chain. Here we report the preparation and characterization of a subcomplex of complex I selectively lacking the flavoprotein part of the N-module. Removing the 51-kDa and the 24-kDa subunit resulted in loss of catalytic activity. The redox centers of the subcomplex could be reduced neither by NADH nor NADPH demonstrating that physiological electron input into complex I occurred exclusively via the N-module and that the NADPH binding site in the 39-kDa subunit and further potential nucleotide binding sites are isolated from the electron transfer pathway within the enzyme. Taking advantage of the selective removal of two of the eight iron–sulfur clusters of complex I and providing additional evidence by redox titration and site-directed mutagenesis, we could for the first time unambiguously assign cluster N1 of fungal complex I to mammalian cluster N1b.

Keywords: Complex I; NADH:ubiquinone oxidoreductase; Subcomplex; *Yarrowia lipolytica*; Iron–sulfur cluster; N-module; NADH binding

1. Introduction

Proton pumping NADH:ubiquinone oxidoreductase is a very large and complex membrane protein with a central function in energy metabolism [1]. The enzyme which is called complex I in mitochondria and NDH-1 in prokaryotes has an L-shaped structure with a peripheral arm and a membrane arm. Recently, the X-ray structure of the peripheral arm of NDH-1 from *Thermus thermophilus* was solved at 3.3 Å resolution [2]. The structure of the complete enzyme was determined using electron microscopy. Reaching 16.5 Å resolution, the 3D reconstruction of complex I from *Yarrowia lipolytica* is the most detailed structure of the holo-enzyme available to date [3].

Bacterial NDH-1 is considered as the minimal form of the enzyme and consists of 14 subunits [4] that confer all bioenergetic functions and are also present in all mitochondrial enzymes. These "central" subunits can be grouped into three functional modules [5]. The N-module consisting of the 75-kDa, the 51-kDa and the 24-kDa subunits comprises the NADH-binding and oxidation site. The Q-module contains the ubiquinone reduction site while the P-module pumps protons across the membrane.

Apart from the central subunits, mitochondrial complex I contains a number of additional subunits with largely unknown function [6,7]. One of these "accessory" subunits is known to bind NADPH [8]. Moreover, it was shown that several subunits of complex I can be labelled by radioactive NAD(H), NADP(H) or ADP derivatives [9]. This raises the question whether the more complicated mitochondrial enzyme contains additional electron input routes. In the mammalian complex, the part containing just the 51-kDa and the 24-kDa subunit and at least one additional accessory subunit has been addressed as the flavoprotein (FP) of complex I [10]. To test whether the flavoprotein part of the N-module is in fact the only electron input device of complex I we have generated and characterized

Abbreviations: DBQ, *n*-decylubiquinone; dSDS-PAGE, doubled sodium dodecylsulfate polyacrylamide gel electrophoresis; DQA, 2-decyl-4-quinazolinyl amine; EPR, electron paramagnetic resonance; HAR, hexaammineruthenium (III); MES, 2-(N-Morpholino)-ethanesulfonic acid; Tris, Tris(hydroxymethyl) aminomethane

* Corresponding author. Universität Frankfurt, Zentrum der Biologischen Chemie, Molekulare Bioenergetik, Theodor-Stern-Kai 7, Haus 26, D-60590 Frankfurt am Main, Germany. Tel.: +49 69 6301 6926; fax: +49 69 6301 6970.
E-mail address: brandt@zbc.kgu.de (U. Brandt).

0005-2728/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2007.03.005

a subcomplex selectively lacking the 51-kDa and 24-kDa subunits.

2. Materials and methods

2.1. Complex I and subcomplex preparation

Native complex I was purified as described [11]. Briefly, the supernatant of solubilized mitochondrial membranes was applied to a Ni-NTA Sepharose column and complex I was eluted in 400 mM NaCl, 140 mM imidazole, 0.1% laurylmaltoside, 20 mM Na-phosphate pH 7.2. Pooled fractions were concentrated using spin devices (cut off M_r 50 000) and applied to a TSK 4000SW size exclusion column operated by an Äkta basic system (GE Healthcare). The subcomplex was generated by shock freezing of the concentrated pool from the Ni-NTA sepharose column in liquid nitrogen. After thawing size exclusion chromatography was carried out as described and only peak fractions were used for further analysis.

2.2. Electrophoresis and densitometry

For separation of complex I subunits, two different gel systems were used. Resolution in the higher molecular mass range was achieved with a Laemmli gradient gel (1 cm 10% acrylamide step on top of a 12–18% acrylamide gradient gel; [12]). For resolution of hydrophobic subunits and subunits in the lower molecular mass range dSDS PAGE was employed [13]. On each gel 20 µg of protein were loaded per sample and densitometry was carried out using a BioRad Chemidoc gel documentation system.

2.3. Strains and site-directed mutagenesis

Y. lipolytica deletion strain *nuam*Δ described earlier [14] was transformed with a replicative plasmid with or without point mutations. All point mutations were generated in *Escherichia coli* by PCR mutagenesis. Mutations R182C, R182H, R182K and R182L were introduced into plasmid pUB4-NUAM that contained a 5.2-kb *Sal*I fragment comprising the *NUAM* gene locus that encodes the 75-kDa subunit of complex I in *Y. lipolytica*. After transformation into *Y. lipolytica* strain *nuam*Δ, plasmids were recovered and the entire NUAM open reading frame was sequenced to verify the introduced point mutations and to check for other sequence changes. It turned out that the pUB4-NUAM plasmid used for mutagenesis and all plasmids derived from this plasmid carried an A559V mutation in the poorly conserved C-terminus of the 75-kDa subunit. We could not find any difference between the *nuam*Δ deletion strain complemented with the plasmid carrying the A559V mutation and the wild type NUAM-gene.

2.4. FMN determination and catalytic activity

FMN was extracted and quantified according to [15]. Enzymatic activities were measured as described [11,16,17]. The pH optimum of the reaction with NADPH was determined and the buffer composition changed accordingly to achieve maximal rates. Na^+/Mes buffer pH 5.5 was used for the NADPH:HAR and Na^+/Mes buffer pH 6.0 for the NADPH:DBQ reaction.

2.5. EPR spectroscopy

X-band EPR spectra were obtained with a Bruker ESP 300E spectrometer equipped with a frequency counter (HP 53159 A, Hewlett Packard), ER 035 M NMR gaussmeter (Bruker, BioSpin) and a liquid helium continuos flow cryostat (Oxford Instruments). EPR spectra were recorded using the following parameters: microwave frequency 9.47 GHz, modulation amplitude 0.64 mT, modulation frequency 100 kHz (all other parameters are given in figure legends). When preparing EPR samples from subcomplex usually 100 µl purified protein (10 mg/ml) were pre-incubated with 5 mM NADH for 30 s. The solution was then transferred into an argon flushed EPR tube already containing 12 µl sodium dithionite (50 mM). After further 20 s incubation the solution was frozen and stored in liquid nitrogen until EPR measurement. A highly concentrated sample (87 mg/ml) of native complex I reduced by 5 mM NADH was taken as a reference. Analysis of EPR data was performed using Winepr® and Simfonia® software from Bruker Biospin.

2.6. Redox titration of cluster N1

Mitochondrial membranes (23 mg/ml protein) from *Y. lipolytica* were dissolved in 50 mM KCl, 100 mM Tris/Cl, pH 7. Redox titration, EPR measurements and data analysis were done as described earlier [18] with the exception that EPR spectra were recorded at 40 K in order to detect cluster N1, S1 and the Rieske iron–sulfur cluster. After subtraction of spectral contributions of the Rieske and S1 cluster, both of which are completely reduced at more positive potential, relative cluster N1 intensities were estimated by comparison with an experimental N1 spectrum obtained from purified wild-type complex I.

3. Results and discussion

3.1. Generation of a subcomplex of complex I lacking the flavoprotein part of the N-module

In the past many protocols were published to split complex I into smaller fragments [6,19–21]. Typically this was achieved by treating the membrane protein complex with harsh detergents or chaotropic agents. In this study we have used a comparably gentle approach employing a single freeze/thaw step in the presence of 400 mM sodium chloride and 140 mM imidazole at neutral pH. These buffer conditions without freezing/thawing are well tolerated by the enzyme and are used in our standard preparation procedure of pure, monodisperse and fully functional complex I [11,16,22]. In the absence of imidazole and at 100 mM sodium chloride and neutral pH it was possible to freeze and thaw the enzyme without significant loss of activity.

In size exclusion chromatography the elution peak of the subcomplex was shifted compared to the native enzyme indicating a reduced mass (Fig. 1). The subcomplex had a somewhat broadened peak profile and an increased tendency to form aggregates. However, the main peak was still symmetrical indicating a monodisperse state of the preparation. For further analysis only the peak fractions were used. Gross conformational changes could be excluded because electron microscopic reconstruction of single particles in 3D revealed that the overall structure of complex I was retained in the subcomplex except that one large domain was missing (Clason,T., Zickermann,V., Ruiz,T., Brandt,U., Radermacher,M. submitted for publication).

The large number of subunits and their distribution over a wide range of molecular masses required the use of two different gel systems to analyse the subunit pattern of the subcomplex. Fig. 2 shows a gradient Laemmli gel with good resolution for the higher molecular weight range. This allowed separation of the 51-kDa and 49-kDa subunits of complex I. The dSDS PAGE shown in Fig. 3 allowed resolution of subunits in the medium to lower mass range and good separation of the hydrophobic subunits which are found above the diagonal of the more hydrophilic subunits [6]. The assignment of subunits has been carried out by N-terminal sequencing [23] and mass spectrometry [6]. SDS-PAGE showed selective depletion of the 51-kDa and 24-kDa subunits and the ST1 protein. The rest of the subunit pattern remained essentially unchanged. The

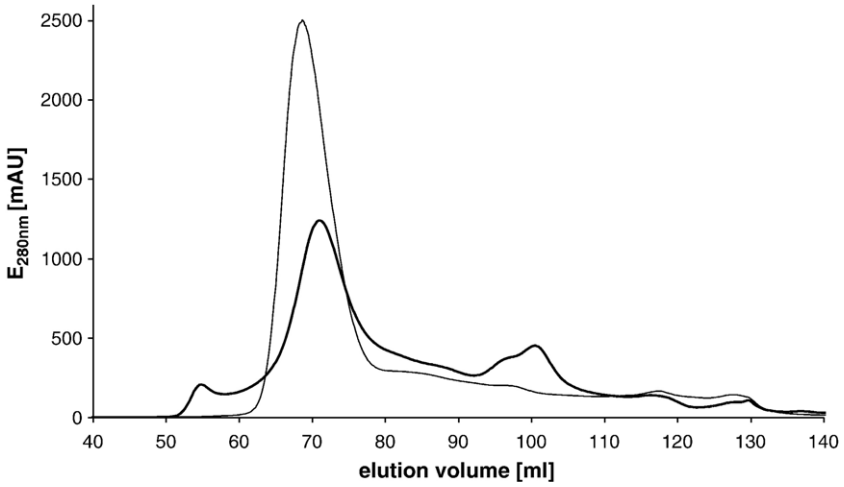

Fig. 1. Elution profile of subcomplex (thick line) and complex I (thin line) from a preparative TSK 4000SW size exclusion column.

preparation did however contain a small amount of holo complex I; the residual content of the 51-kDa band was estimated by densitometric analysis to be less than 20% (Fig. 2).

Complex I from *Y. lipolytica* contains an active sulfurtransferase (rhodanese) in substoichiometric amounts [24]. This protein with a mass of 34.6 kDa (ST1 in Figs. 2 and 3) was completely lost during generation of the subcomplex. This was not unexpected because this protein is only loosely associated with complex I [24]. Deletion of the ST1 gene has no impact on complex I activity or assembly and the iron–sulfur clusters are unchanged in their EPR properties [24]. Therefore, the absence of this protein was not relevant in the context of this study and needs not to be discussed further here. In fact, subcomplex prepared from an ST1 deletion strain was indistinguishable from that prepared from the wild-type strain (not shown).

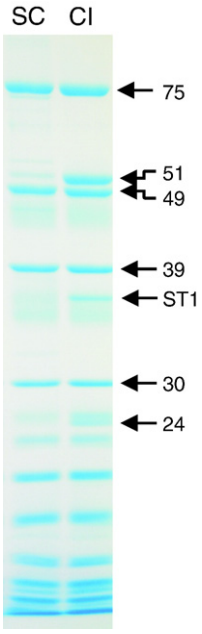

Fig. 2. Gradient SDS PAGE of purified subcomplex (SC) and complex I (CI) from *Y. lipolytica*. The 51-kDa subunit is clearly depleted and the sulfurtransferase (ST1) present in native complex I is lacking in the subcomplex. Depletion of the 24-kDa subunit is apparent but resolution of subunits with a molecular mass smaller than 30-kDa is superior in the dSDS PAGE presented in Fig. 3.

The 51-kDa subunit contains the binding sites for NADH and the primary electron acceptor FMN [25] and is tightly associated with the 24-kDa subunit. Together with the 75-kDa subunit these two subunits form the N-module of complex I [1] and contain binding motifs for iron–sulfur clusters (see below). Earlier work on subcomplexes [10] and the recent X-ray structure of the hydrophilic part of complex I [2] show that the 51-kDa and 24-kDa subunits form a distinct structural entity which can be addressed as the flavoprotein part of complex I. It should be noted that the 10-kDa subunit found in the bovine flavoprotein fragment is not conserved in *Y. lipolytica*. In summary, the subcomplex described here that was prepared by a single freeze/thaw step allowed us to study complex I that selectively lacked its NADH binding and electron entry domain.

3.2. FMN content and complex I activity

A stoichiometry of 1.02 mole FMN per mole complex I was determined for the native enzyme. In two batches of subcomplex a stoichiometry of 0.11 and 0.14 mole FMN per mole subcomplex was determined. The decrease in FMN content was in good agreement with the depletion of 51-kDa subunit as determined by gel-densitometry. This strongly argues against the presence of a second FMN molecule in a second binding site of the Q-module as proposed by Albracht and colleagues [26].

Complex I prepared from *Y. lipolytica* mitochondria exhibited a high specific NADH: decylubiquinone oxidoreductase activity of 6.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Consistent with the removal of most of the flavoprotein part of the N-module, this activity was reduced to 7% in the subcomplex (Table 1). As ubiquinone reductase activity may also become reduced by delipidation [16] or general damage of the complex, we also measured electron transfer from NADH to the artificial electron acceptor hexaammineruthenium(III) (HAR) that is known to require only the 51-kDa subunit [27]. This activity was also

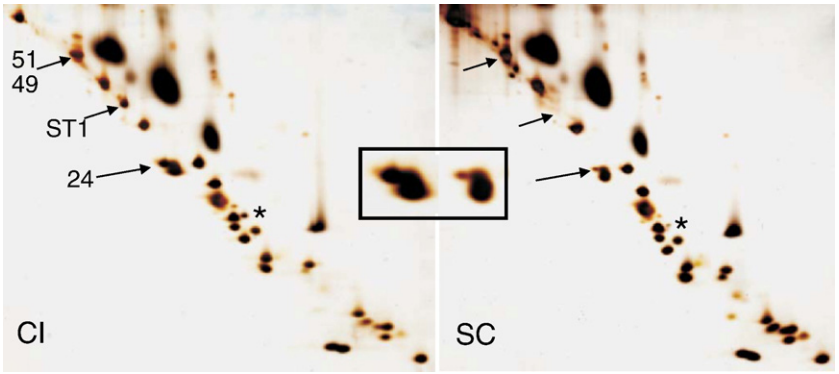

Fig. 3. dSDS PAGE of purified subcomplex (SC) and complex I (CI). Arrows indicate the position of the 51-kDa and 49-kDa subunits, sulfurtransferase (ST1) and the 24-kDa subunit. The inset shows the depletion of the 24-kDa subunit in detail. The 51-kDa and 49-kDa subunits of complex I could not be separated on this type of gel. The asterisk marks a spot not present in most complex I preparations that was therefore considered an impurity. Assignment of subunits according to [6].

reduced to 6% in the subcomplex (Table 1) providing further evidence that about 90% of the complexes had lost the flavoprotein part of the N-module.

Complex I can accept electrons not only from NADH but also from NADPH. The pH optimum for NADPH is more acidic and the maximal rates were only about 4% of those with NADH (Table 1). It has been discussed whether electron input into the enzyme from NADPH occurs via the same site as for NADH or if there are alternative routes. In fact, an NADPH binding site was found in the 39-kDa subunit of complex I [8,28] and it was reported that also other complex I subunits can be labelled by radioactive nicotinamide–nucleotide derivatives [9]. The NADPH:HAR activity of the subcomplex lacking the flavoprotein part of the N-module was reduced by about the same extent as the NADH-dependent activities and NADPH:DBQ activity was below the detection limit (Table 1). This suggested that the NADPH-dependent activities observed in the native enzyme were entirely due to the reaction with this site and that alternate nucleotide binding sites could not feed electrons into complex I. All catalytic activities of the subcomplex were even somewhat lower than expected from the residual level of the 51-kDa subunit as judged by densitometric analysis of SDS-gels. Therefore, the catalytic activity in the subcomplex preparation can be completely accounted for by the presence of small residual amounts of native enzyme.

3.3. EPR spectroscopy

In native complex I from *Y. lipolytica* one binuclear and four tetranuclear iron–sulfur clusters can be detected by EPR spectroscopy. In addition, one binuclear and two tetranuclear clusters are present but cannot be detected by EPR spectroscopy. Without addition of reductant the subcomplex preparation gave only very small EPR signals probably due to some contaminations with other respiratory complexes, mainly complex II with a characteristic $g=2.02$ signal in the oxidized state (Fig. 4A). Upon incubation of subcomplex with NADH, minor EPR signal intensities typical for complex I were detectable (Fig. 4B). Complete reduction of redox centers in the subcomplex was achieved by adding dithionite (Fig. 4C), but the EPR signals of iron–sulfur cluster N3 were missing (Fig. 4C and D), as had been expected from the well established location of this redox center in the 51-kDa subunit [29,30]. No residual signal from cluster N3 could be detected due to overlapping spectra of other clusters with much higher intensity. However, based on the cluster N2 signal intensities (Fig. 4B and C) we estimated that ~8% of cluster N2 was reduced by NADH matching the residual catalytic activity originating from the remaining fraction of intact complex I. NADPH, like NADH induced virtually no reduction of the iron–sulfur clusters in the subcomplex while complete reduction could be observed in the native enzyme (data not shown). This result corresponded well with the loss of catalytic activity in the subcomplex and indicated that the EPR visible redox centers of complex I were inaccessible from the 39-kDa subunit and other putative NAD(P)H binding sites. We concluded that the NADH acceptor site had been removed upon formation of the subcomplex and that there were no alternative NADH or NADPH binding sites which would have allowed reduction of the iron–sulfur clusters of complex I.

Table 1
Catalytic activities of complex I and the subcomplex

Activity	Native enzyme	Subcomplex	%
	$\mu mol \cdot min^{-1} \cdot mg^{-1}$		
NADH-HAR	58	3.6	6
NADH-DBQ	6.3	0.45	7
NADPH-HAR	2.2	0.12	6
NADPH-DBQ	0.24	n.d. [a]	–

[a] Not detectable.

3.4. Assignment of iron–sulfur cluster N1 of Y. lipolytica complex I

In mammalian complex I two binuclear clusters N1a and N1b can be detected [31]. Like in other fungi, only one of them is EPR detectable in complex I from *Y. lipolytica* and it has not been demonstrated unambiguously whether this cluster corresponds to mammalian cluster N1a or N1b. Because mammalian cluster N1a had been shown to be located in the 24-kDa subunit and cluster N1b in the 75-kDa subunit, we expected to get a

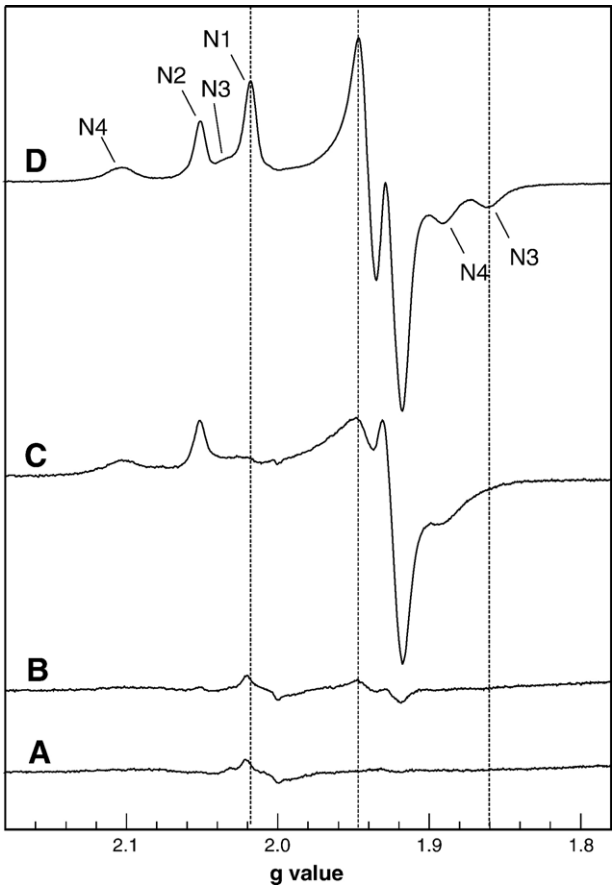

Fig. 4. EPR spectra of native complex I and subcomplex at 12 K. (A) The subcomplex preparation without added reductant showed only minor EPR signals mainly originating from complex II contaminations. (B) Upon addition of 5 mM NADH typical complex I signals appeared but at very low intensity. (C) Reduction of subcomplex with 5 mM NADH and 5 mM dithionite resulted in pronounced EPR signals from clusters N2 and N4 but signals from cluster N1 had changed dramatically and those from cluster N3 were absent. (D) Native complex I reduced by 5 mM NADH. Representative EPR signals for individual iron–sulfur clusters are indicated. Dotted lines designate characteristic signals from clusters N1 and N3 which changed or disappeared in the subcomplex spectrum (C). Microwave power, 1 mW, protein concentration: native complex I 87 mg/ml, subcomplex 10 mg/ml.

clear answer to this question from our subcomplex preparation. However the situation turned out to be more complicated.

At first sight it seemed that the EPR spectrum of cluster N1 was not present in the subcomplex lacking the 24-kDa subunit (Fig. 4C). However, closer inspection revealed that it had only changed dramatically: at 12 K the g_z signal was hardly detectable and the g_{xy} region had become lower due to significant broadening. The situation became clearer at 25 K where only clusters N1 and N2 were visible (Fig. 5) and at 40 K where only binuclear clusters are detectable (Fig. 6). The ratio of clusters N1/N2 was estimated from EPR spectra at 25 K. The intensity of N2 was obtained by double integration of a simulated N2 spectrum adjusted to the experimental spectrum. This simulation was subtracted from the 25 K spectrum. Double integration of the resulting difference spectrum yielded the intensity of the binuclear cluster. Comparison of signal intensity ratios from cluster N1 and cluster N2 in the native complex and in the subcomplex revealed that they were still present in a 1:1 ratio. This indicated that the binuclear cluster was not lost during the fragmentation procedure and we concluded that it must correspond to iron–sulfur cluster N1b.

The marked spectral differences indicated significant changes in the environment of the binuclear cluster that could be easily explained based on the X-ray structure of the peripheral arm (Fig. 7; [2]). Cluster N1b is close to the interface of the 75-kDa and the 51-kDa subunits and the edge-to-edge distance of clusters N3 and N1b is only 11 Å. Therefore it can be expected that after removal of the 51-kDa and the 24-kDa subunit the binuclear cluster in the 75-kDa subunit becomes more exposed to the solvent which may have led to the observed spectral changes. The selective lack of cluster N1b insertion into the 75-kDa subunit of *E. coli* expressed as a separate protein further highlights the susceptibility of this binuclear cluster [32].

Additional support for the assignment of the binuclear clusters came from a more detailed analysis of iron–sulfur cluster N1 of *Y. lipolytica* complex I. We determined a redox midpoint potential at pH 7 of −262 mV for the binuclear cluster in *Y. lipolytica* complex I (data not shown) which also favoured the idea that the EPR signal detectable at 40 K (g_z=2.018, g_{xy}=1.941) resulted from cluster N1b (Fig. 6, B) because

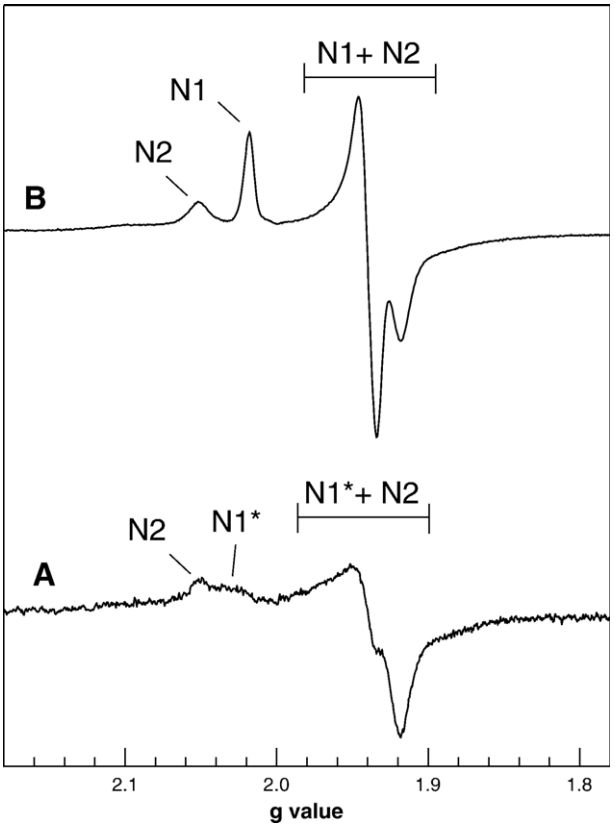

Fig. 5. EPR spectra of subcomplex (A) and native complex I (B) at 25 K. At 25 K only EPR signals from cluster N2 and N1 are detectable. Due to fast spin relaxation the EPR signal of cluster N2 became broadened and the signals of clusters N3 and N4 were not detectable under these conditions. Modified cluster N1 is marked by an asterisk. Samples were the same as in Fig. 4; microwave power, 2 mW.

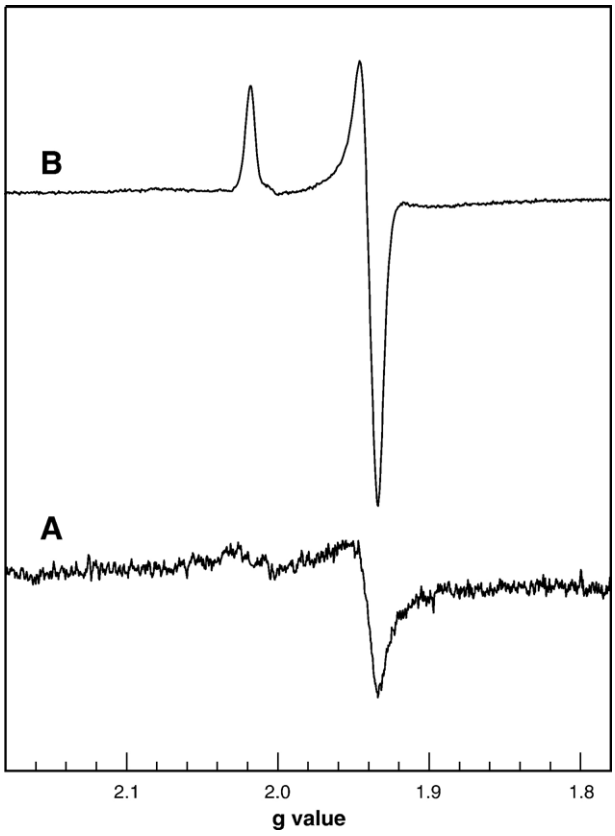

Fig. 6. EPR spectra of subcomplex (A) and native complex I (B) at 40 K. At 40 K only binuclear clusters are detectable. Disruption of complex I results in a shifted and broadened EPR spectrum of iron–sulfur cluster N1. Samples were the same as in Fig. 4; microwave power, 1 mW.

cluster N1a is expected to have a much more negative redox midpoint potential [33]. Moreover, site-directed mutagenesis of position R182 in the 75-kDa subunit of complex I in *Y. lipolytica* provided additional evidence that the EPR detectable

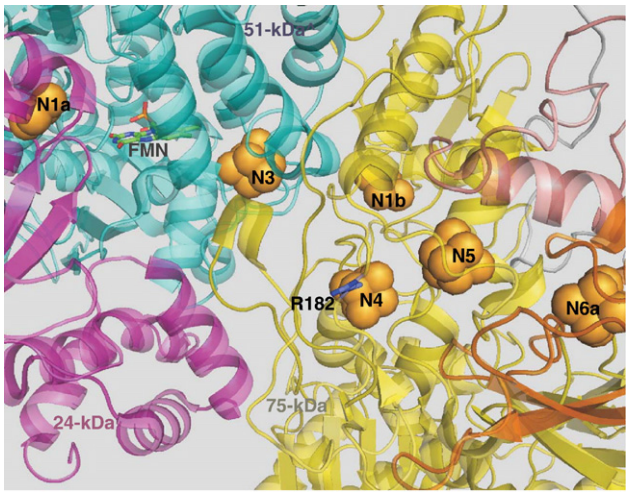

Fig. 7. Interface region between the 51-kDa and the 75-kDa subunits of complex I. The residue corresponding to R182 in *Y. lipolytica* is shown as stick model in blue. The figure was created using the coordinates of the peripheral arm of *Th. thermophilus* complex I (2FUG; [2]) and the Pymol software package version 0.99.

binuclear cluster of *Y. lipolytica* complex I corresponded to mammalian cluster N1b. R182 is located in a loop that is in contact with the domain ligating the binuclear cluster in the 75-kDa subunit (Fig. 7). EPR spectra recorded at 40 K (Fig. 8), where only signals originating from the slow-relaxing binuclear iron–sulfur cluster N1 contribute to the EPR spectrum, revealed that the g_z signal of cluster N1 was significantly decreased and shifted in complex I harboring a R182H point mutation and even more if R182 was mutated to leucine. Moreover, in mutant R182L the g_{xy} signal was shifted significantly to a higher g value and the intensity of the spectrum was reduced to 60–70% of the parental strain. In the spectra of mutants R182C and R182K no significant shifts were observed. It should be noted that we did not find any changes in the EPR signature of the tetranuclear clusters, especially of cluster N4 which resides close to R182 in the 75-kDa subunit. All mutants exhibited unchanged or moderately reduced catalytic activities and we found no significant difference in the midpoint potential of

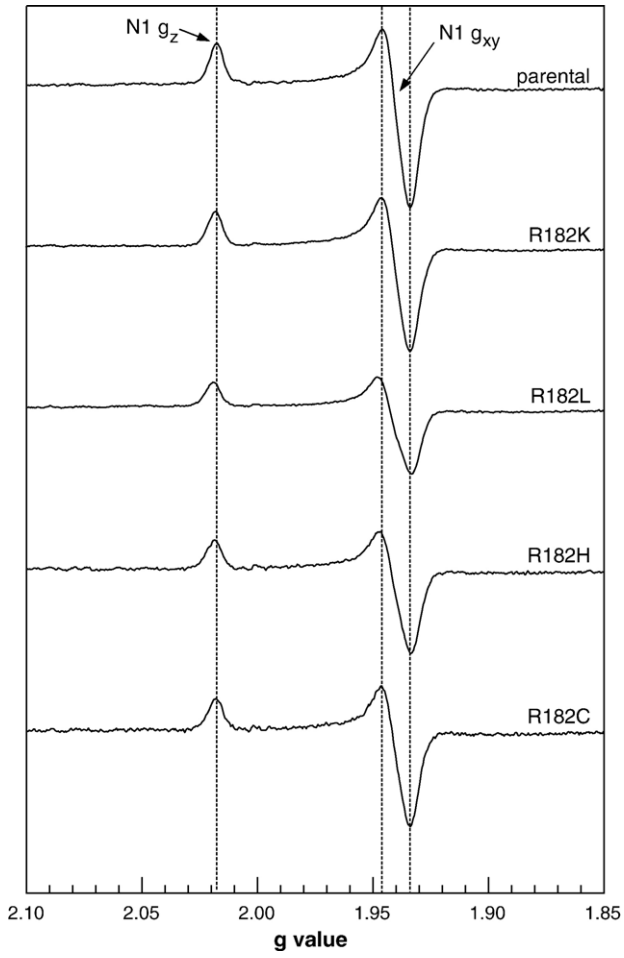

Fig. 8. EPR spectra of purified complex I recorded at 40 K and 5 mW microwave power. Complex I samples were reduced with 2 mM NADH and 5 mM sodium dithionite. Spectra were normalized to the N2 signal recorded at 12 K (protein concentrations of EPR samples: parental 10 mg/ml; R182K 12.5 mg/ml; R182L 10 mg/ml; R182H 6.7 mg/ml and R182C 4.2 mg/ml). Vertical dashed lines indicate the position of the maxima and minima in the spectrum of complex I from the parental strain. Significant changes in signal intensities and signal positions of iron–sulfur cluster N1 can be seen, particularly in complex I from mutant R182L.

cluster N1 between parental strain (E_{m7} = −262 mV) and mutant R182L (E_{m7} = −245 mV) complex I (data not shown).

4. Conclusions

The subcomplex preparation of mitochondrial complex I characterized here selectively lacked the flavoprotein part of the N-module and contained only residual amounts of intact complex I. Upon removal of the 51-kDa and the 24-kDa subunits catalytic activity was lost and the redox centers of complex I could not be reduced by NADH or NADPH anymore. This demonstrated that physiological electron input into complex I occurs exclusively via the N-module and that the NADPH binding site in the 39-kDa subunit is isolated from the electron transfer pathway within the enzyme.

As one of the two binuclear clusters is bound to the 24-kDa subunit of complex I, EPR spectroscopic analysis of the subcomplex for the first time provided direct evidence for the identity of the only EPR detectable binuclear iron–sulfur cluster of fungal complex I. The assignment of cluster N1 of *Y. lipolytica* complex I to mammalian cluster N1b was backed up further by determining the redox midpoint potential of cluster N1 and by site-directed mutagenesis.

Acknowledgements

We thank Gudrun Beyer, Andrea Duchene, and Karin Siegmund for excellent technical assistance and Ilka Wittig and Herrmann Schägger for helpful discussions on the electrophoresis part of this study. This work was supported by the Deutsche Forschungsgemeinschaft SFB472 Project P2.

References

[1] U. Brandt, Energy converting NADH:quinone oxidoreductases, Annu. Rev. Biochem. 75 (2006) 69–92.
[2] L.A. Sazanov, P. Hinchliffe, Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*, Science 311 (2006) 1430–1436.
[3] M. Radermacher, T. Ruiz, T. Clason, S. Benjamin, U. Brandt, V. Zickermann, The three-dimensional structure of complex I from *Yarrowia lipolytica*: a highly dynamic enzyme, J. Struct. Biol. 154 (2006) 269–279.
[4] T. Friedrich, K. Steinmüller, H. Weiss, The proton-pumping respiratory complex I of bacteria and mitochondria and its homologue in chloroplasts, FEBS Lett. 367 (1995) 107–111.
[5] U. Brandt, S. Kerscher, S. Dröse, K. Zwicker, V. Zickermann, Proton pumping by NADH:ubiquinone oxidoreductase. A redox driven conformational change mechanism? FEBS Lett. 545 (2003) 9–17.
[6] A. Abdrakhmanova, V. Zickermann, M. Bostina, M. Radermacher, H. Schägger, S. Kerscher, U. Brandt, Subunit composition of mitochondrial complex I from the yeast *Yarrowia lipolytica*, Biochim. Biophys. Acta 1658 (2004) 148–156.
[7] J. Carroll, I.M. Fearnley, R.J. Shannon, J. Hirst, J.E. Walker, Analysis of the subunit composition of complex I from bovine heart mitochondria, Mol. Cell Proteomics 2 (2003) 117–126.
[8] A. Abdrakhmanova, K. Zwicker, S. Kerscher, V. Zickermann, U. Brandt, Tight binding of NADPH to the 39-kDa subunit of complex I is not required for catalytic activity but stabilizes the multiprotein complex, Biochim. Biophys. Acta 1757 (2006) 1676–1682.
[9] M. Yamaguchi, G. Belogrudov, A. Matsuno-Yagi, Y. Hatefi, The multiple nicotinamide nucleotide-binding subunits of bovine heart mitochondrial NADH:ubiquinone oxidoreductase (complex I), Eur. J. Biochem. 267 (2000) 329–336.
[10] Y.M. Galante, Y. Hatefi, Resolution of complex I and isolation of NADH dehydrogenase and an iron–sulfur protein, Methods Enzymol. 53 (1978) 15–21.
[11] N. Kashani-Poor, S. Kerscher, V. Zickermann, U. Brandt, Efficient large scale purification of his-tagged proton translocating NADH:ubiquinone oxidoreductase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*, Biochim. Biophys. Acta 1504 (2001) 363–370.
[12] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[13] I. Rais, M. Karas, H. Schägger, Two-dimensional electrophoresis for the isolation of integral membrane proteins and mass spectrometric identification, Proteomics 4 (2004) 2567–2571.
[14] A. Waletko, K. Zwicker, A. Abdrakhmanova, V. Zickermann, U. Brandt, S. Kerscher, Histidine 129 in the 75-kDa subunit of mitochondrial complex I from *Yarrowia lipolytica* is not a ligand for [Fe4S4] cluster N5 but is required for catalytic activity, J. Biol. Chem. 280 (2005) 5622–5625.
[15] J.G. Okun, V. Zickermann, K. Zwicker, H. Schägger, U. Brandt, Binding of detergents and inhibitors to bovine complex I—A novel purification procedure of bovine complex I retaining full inhibitor sensitivity, Biochim. Biophys. Acta 1459 (2000) 77–87.
[16] S. Dröse, K. Zwicker, U. Brandt, Full recovery of the NADH:ubiquinone activity of complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica* by the addition of phospholipids, Biochim. Biophys. Acta 1556 (2002) 65–72.
[17] L. Grgic, K. Zwicker, N. Kashani-Poor, S. Kerscher, U. Brandt, Functional significance of conserved histidines and arginines in the 49 kDa subunit of mitochondrial complex I, J. Biol. Chem. 279 (2004) 21193–21199.
[18] A. Garofano, K. Zwicker, S. Kerscher, P. Okun, U. Brandt, Two aspartic acid residues in the PSST-homologous NUKM subunit of complex I from *Yarrowia lipolytica* are essential for catalytic activity, J. Biol. Chem. 278 (2003) 42435–42440.
[19] Y.M. Galante, Y. Hatefi, Purification and molecular and enzymatic properties of mitochondrial NADH dehydrogenase, Arch. Biochem. Biophys. 192 (1979) 559–568.
[20] M. Finel, J.M. Skehel, S.P.J. Albracht, I.M. Fearnley, J.E. Walker, Resolution of NADH:ubiquinone oxidoreductase from bovine heart mitochondria into two subcomplexes, one of which contains the redox centers of the enzyme, Biochemistry 31 (1992) 11425–11434.
[21] L.A. Sazanov, S.Y. Peak-Chew, I.M. Fearnley, J.E. Walker, Resolution of the membrane domain of bovine complex I into subcomplexes: implications for the structural organization of the enzyme, Biochemistry 39 (2000) 7229–7235.
[22] A. Galkin, S. Dröse, U. Brandt, The proton pumping stoichiometry of purified mitochondrial complex I reconstituted into proteoliposomes, Biochim. Biophys. Acta 1757 (2006) 1575–1581.
[23] R. Djafarzadeh, S. Kerscher, K. Zwicker, M. Radermacher, M. Lindahl, H. Schägger, U. Brandt, Biophysical and structural characterization of proton-translocating NADH-dehydrogenase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*, Biochim. Biophys. Acta 1459 (2000) 230–238.
[24] A. Abdrakhmanova, K. Dobrynin, K. Zwicker, S. Kerscher, U. Brandt, Functional sulfurtransferase is associated with mitochondrial complex I from *Yarrowia lipolytica*, but is not required for assembly of its iron–sulfur clusters, FEBS Lett. 579 (2005) 6781–6785.
[25] S.J. Pilkington, J.M. Skehel, R.B. Gennis, J.E. Walker, Relationship between mitochondrial NADH-ubiquinone reductase and a bacterial NAD-reducing hydrogenase, Biochemistry 30 (1991) 2166–2175.
[26] S.P.J. Albracht, R. Hedderich, Learning from hydrogenases: location of a proton pump and of a second FMN in bovine NADH-ubiquinone oxidoreductase (ComplexI), FEBS Lett. 24275 (2000) 1–6.
[27] E.V. Gavrikova, V.G. Grivennikova, V.D. Sled, T. Ohnishi, A.D. Vinogradov, Kinetics of the mitochondrial three-subunit NADH dehydrogenase interaction with hexammineruthenium(III), Biochim. Biophys. Acta 1230 (1995) 23–30.

[28] I.M. Fearnley, J.E. Walker, Conservation of sequences of subunits of mitochondrial complex I and their relationships with other proteins, Biochim. Biophys. Acta 1140 (1992) 105–134.
[29] W. Fecke, V.D. Sled, T. Ohnishi, H. Weiss, Disruption of the gene encoding the NADH-binding subunit of NADH: ubiquinone oxidoreductase in *Neurospora crassa*. Formation of a partially assembled enzyme without FMN and the iron–sulphur cluster N-3, Eur. J. Biochem. 220 (1994) 551–558.
[30] T. Yano, V.D. Sled, T. Ohnishi, T. Yagi, Expression and characterization of the flavoprotein subcomplex composed of 50-kDa (NQO1) and 5-kDa (NQO2) subunits of the proton-translocating NADH-quinone oxidoreductase of *Pracoccus denitrificans*, J. Biol. Chem. 271 (1996) 5907–5913.
[31] T. Ohnishi, Iron–sulfur clusters semiquinones in complex I, Biochim. Biophys. Acta 1364 (1998) 186–206.
[32] E. Nakamaru-Ogiso, T. Yano, T. Ohnishi, Characterization of the iron–sulfur cluster N7 (N1c) in the subunit NuoG of the proton-translocating NADH-quinone oxidoreductase from *Escherichia coli*, J. Biol. Chem. 280 (2005) 301–307.
[33] Y. Zu, S. Di Bernardo, T. Yagi, J. Hirst, Redox properties of the [2Fe–2S] center in the 24 kDa (NQO2) subunit of NADH:ubiquinone oxidoreductase (Complex I), Biochemistry 41 (2002) 10056–10069.