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Characterization of the NADH:ubiquinone oxidoreductase (complex I) in the trypanosomatid *Phytomonas serpens* (Kinetoplastida)

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NADH dehydrogenase activity was characterized in the mitochondrial lysates of *Phytomonas serpens*, a trypanosomatid flagellate parasitizing plants. Two different high molecular weight NADH dehydrogenases were characterized by native PAGE and detected by direct in-gel activity staining. The association of NADH dehydrogenase activities with two distinct multisubunit complexes was revealed in the second dimension performed under denaturing conditions. One subunit present in both complexes cross-reacted with the antibody against the 39 kDa subunit of bovine complex I. Out of several subunits analyzed by MS, one contained a domain characteristic for the LYR family subunit of the NADH:ubiquinone oxidoreductases. Spectrophotometric measurement of the NADH:ubiquinone 10 and NADH:ferricyanide dehydrogenase activities revealed their different sensitivities to rotenone, piericidin, and diphenyl iodonium.

NADH:ubiquinone oxidoreductase (complex I) of the respiratory chain catalyzes the transfer of an electron pair from NADH to quinone, an oxidation reaction that is coupled with the translocation of four protons across the membrane. In prokaryotes, complex I is composed of 14 subunits, the homologs of which constitute the so-called core of the eukaryotic complex I [1]. In the mitochondrion of some eukaryotes, this has evolved into a complex formed of up to 45 different subunits, thus becoming one of the largest membrane protein complexes known [2]. Furthermore, complex I contains a noncovalently bound flavin mononucleotide and nine iron–sulfur clusters [3]. However, it can also be reduced down to mere two subunits, as is the case for complex I in the hydrogenosome of *Trichomonas vaginalis* [4]. The recently solved crystal structure of the bacterial complex [5] confirmed the predicted characteristic L-shaped structure of both bacterial and mitochondrial enzymes, composed of the hydrophobic and hydrophilic arms [6,7].

Kinetoplastid flagellates such as *Trypanosoma* and *Leishmania* are early-branching eukaryotes responsible for sleeping sickness, Chagas disease, leishmaniases of humans, and nagana of lifestock. Phytomonads are trypanosomatids parasitizing various plants, causing economically important diseases, such as wilts and heartrot disease of coconut and oil palms and coffee in Latin America and the Caribbean. In this study, we have analyzed the function and composition of complex I in *Phytomonas serpens*. The very presence of this

Abbreviations
BN, blue native; CBB, Coomassie Brilliant Blue; DPI, diphenyl iodonium.

complex in the model trypanosomatid *Trypanosoma brucei* and related species is a matter of much debate. Whereas several lines of evidence have proved the presence of active respiratory complexes II–V, direct detection of complex I has remained elusive. Arguments for the existence of complex I in trypanosomatids can be summarized as follows: (a) in *Try. brucei*, mRNAs of putative mitochondrial-encoded subunits undergo developmentally regulated RNA editing in stages in which their presence would be appropriate [8,9]; (b) homologs of several nuclear-encoded subunits are present in the genomes of *Try. cruzi*, *Try. brucei* and *Leishmania tarentolae* [10] (our unpublished results); (c) antibodies against subunits of complex I of other organisms detected putative homologs in the mitochondrial lysates of *Try. brucei* [11,12] and *P. serpens* [13]; and (d) in the mitochondrial lysates of *Try. brucei*, NADH dehydrogenase activity has been detected spectrophotometrically and by in-gel activity staining [14], and found to be inhibited by rotenone, a specific inhibitor of complex I [11,12]. However, serious doubt was cast over the latter evidence, as the concentrations of rotenone used were very high and may have inhibited other electron carriers [15,16]. Another line of evidence indirectly supporting the presence of complex I in both procyclic and bloodstream stages of *Try. brucei* comes from experiments showing constitutive import of the nuclear-encoded subunit ndhK into the mitochondrion [17].

The absence of complex I was originally reported for procyclic *Try. brucei* [18], and later also for the culture forms of *Try. cruzi* [19], and laboratory-cultivated strains of *Crithidia fasciculata* [20] and *L. tarentolae* [21]. It has been proposed that complex I is missing, as its activity was dispensable for cells cultivated in rich media for a prolonged period of time [22]; this conclusion was supported by the absence of translatable mitochondrial mRNAs for complex I subunits [21]. Because complex I subunits in trypanosomatids have only low sequence similarity with their putative homologs in other eukaryotes, it was speculated that, in fact, they may represent subunits of a complex that is rather different from the typical eukaryotic complex I. The detection of complex I in *Try. brucei*, using specific inhibitors, is further hampered by the presence of single-peptide alternative NADH dehydrogenases [23,24], although the latest data indicate that only a single alternative dehydrogenase exists in *Try. brucei* (D. Beattie, personal communication). Moreover, an alternative dehydrogenase was invoked to explain the inhibitory effects of rotenone and atovaquone in *P. serpens* [13]. Therefore, in the absence of direct evidence, it has been unknown whether complex I is present in kinetoplastid flagellates, and if it is, what its functions are. Herein, we provide several lines of evidence for the presence of complex I in *P. serpens*.

Results

NADH dehydrogenase activity in the mitochondrial lysate of *P. serpens* was assayed by spectrophotometry using two different electron acceptors and three inhibitors. The employed electron acceptors were ubiquinone 10, an analog of natural ubiquinone that binds to the region of the 49 kDa and PSST subunits [25], and ferricyanide, for which binding to the hydrophilic peripheral arm protruding into the matrix has been predicted for bovine complex I [26]. Piericidin and rotenone represent specific inhibitors of complex I [27,28], whereas diphenyl iodonium (DPI) irreversibly binds flavins, and was used for inhibition of the alternative NADH dehydrogenase [23,24,29]. We have tested the predicted specific inhibitory effect of DPI for the single-peptide alternative NADH dehydrogenase using the yeast *Yarrowia lipolytica*, in which its presence along with complex I has been well documented [30]. Blue native (BN) 2–15% gradient gel electrophoresis and subsequent in-gel activity staining of the mitochondrial lysate of *Y. lipolytica* confirmed that DPI targets only the alternative enzyme (Fig. 1A).

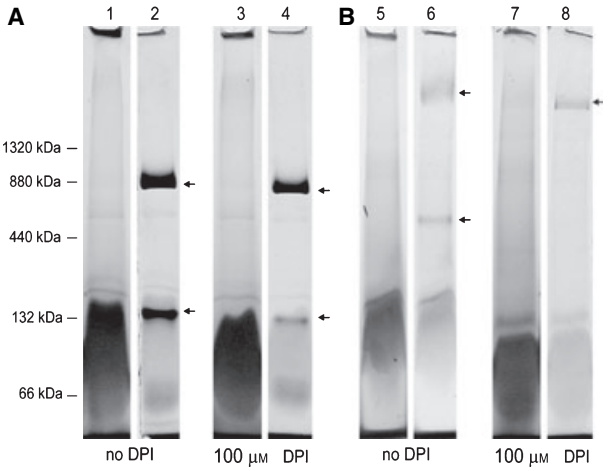

Fig. 1. In-gel staining of the NADH dehydrogenase activity in *Y. lipolytica* (A) and *P. serpens* (B). Electrophoresis was performed in 2–15% BN gradient gel. Lanes 1, 3, 5 and 7: BN gel photographed immediately after the run. Lanes 2, 4, 6 and 8: BN gel after in-gel activity staining without (lanes 2 and 6) and with (lanes 4 and 8) 100 μM DPI. Arrows point to complexes with NADH dehydrogenase activity. The size of the lower band with NADH dehydrogenase activity in lanes 2 and 4 corresponds to a dimer of the alternative dehydrogenase of *Y. lipolytica* (molecular mass of monomer is 67 kDa). The position of molecular mass markers is indicated on the left (BSA and ferritin).

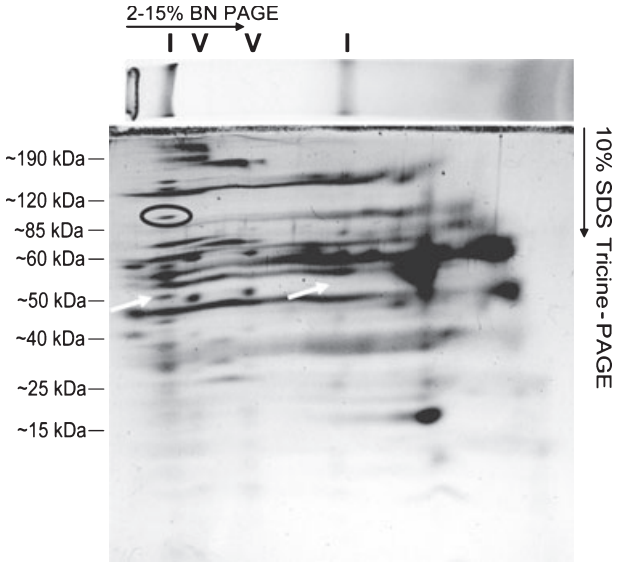

Fig. 2. Mitochondrial lysate of *P. serpens* was resolved in two-dimensional (2–15% gradient BN/10% Tricine-SDS) gel and stained with CBB. The positions of respiratory complexes I and V detected by in-gel activity staining (shown only for complex I) are indicated by Roman numerals. The sequenced subunit is shown by an ellipsis; arrows point to a unique subunit present only in the large form of complex I. The position of molecular mass markers is indicated on the left.

When the mitochondrial lysate of *P. serpens* was resolved under the same conditions, a previously reported [14] strong signal was visualized at about 650–700 kDa, and, unexpectedly, another specific and even stronger band appeared higher up in the gel (Fig. 1B, lane 6). A subsequent incubation of the gel in DPI resulted in inhibition of the lower band only (Fig. 1B, lane 8). On the basis of the size of multimers of ferritin used as a molecular marker, the size of the upper band was estimated to be about 2.2 MDa. However, size inferred from electrophoretic migration can be misleading, as multimerization frequently occurs in the BN gels [22]. To assess this possibility in *P. serpens*, we resorted to two-dimensional gel analysis, with the first and second dimensions performed in a 2–15% gradient BN gel and a 10% denaturing gel, respectively. A representative two-dimensional gel of *P. serpens* aligned with the in-gel-stained first dimension for orientation is shown in Fig. 2. Several proteins are visible in regions of the second dimension that correspond to the activity bands in the BN gel. Five protein spots from the upper band, corresponding to the high molecular mass region, were excised from a typical two-dimensional gel, in-gel digested with trypsin, and subjected to MS analysis. Only the protein highlighted in Fig. 2 was clearly identified by the SEQUEST software (Fig. 3 shows an MS/MS spectrum identifying the oligopeptide EQLFQYLLR). The six identified peptides were matched to different regions of a single hypothetical protein conceptually translated from the *L. major*, *Try. cruzi* and *Try. brucei* genomes (Fig. 4A). In order to increase the sequence coverage of this hit and to identify proteins in the other spots, we derived several partial amino acid sequences by *de novo* sequencing. However, our attempts to match these peptides using different BLAST algorithms to any known sequence were unsuccessful.

This gene, highly conserved among trypanosomatids and annotated as 'hypothetical', shows significant similarity to members of the complex I LYR protein

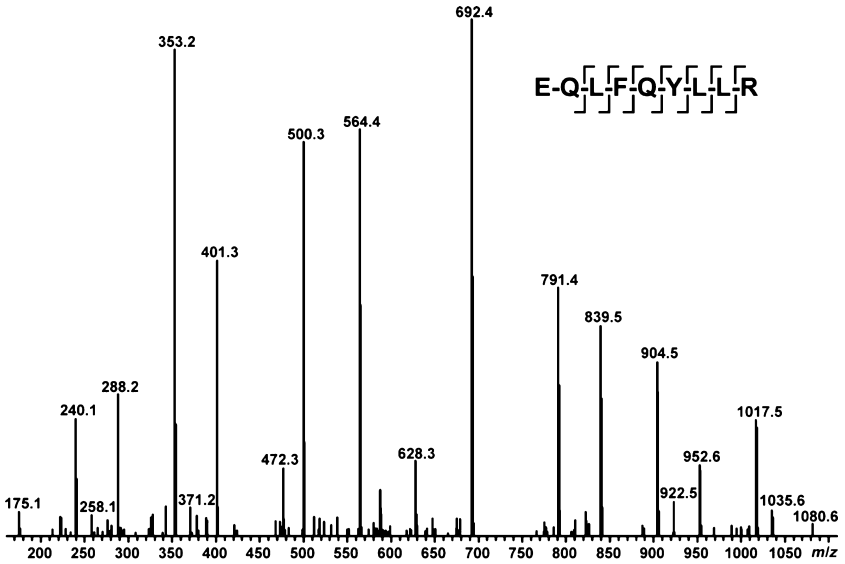

Fig. 3. MS/MS spectrum identifying the oligopeptide EQLFQYLLR. Nearly complete b-ion and y-ion series were found as indicated by the ticks in the insert in the top right corner.

Fig. 4. Sequence analysis of the LYR family subunit of complex I in *P. serpens*. (A) Alignment of the identified sequences from *P. serpens* with closely related sequences from *Try. brucei* (XP_827964), *Try. cruzi* (XP_812266) and *L. major* (CAJ07108). In the identified sequences from *P. serpens*, L stands for Leu/Ile. All peptides fit into the central region of these proteins (all are annotated as hypothetical) and show a very high degree of similarity. (B) Multiple alignment of selected members of the LYR protein family: *Try. brucei* (XP_827964), *Try. cruzi* (XP_812266), *L. major* (CAJ07108), *Xenopus laevis* (AAH88949), *Rattus norvegicus* (XP_235518), *Bos taurus* (AAI02431) and *Homo sapiens* (CAI19953). Fully conserved residues are highlighted in bold and indicated by '*'; ':' and '.' indicate conserved 'strong' and 'weaker' groups, respectively, according to CLUSTALX.

family (accession number PF05347), and in particular with subunit B14 of NADH:ubiquinone oxidoreductases of several eukaryotes (Fig. 4B). This family includes small subunits of complex I, for which the presence of the LYR tripeptide in the N-terminal part is characteristic. Interestingly, the size of the B14 subunit in most eukaryotes is ~15 kDa, whereas its predicted size in trypanosomatids varies from 77 to 83 kDa, with the *P. serpens* homolog also falling into this range, as judged from its mobility (Fig. 2).

This analysis confirmed that in *P. serpens*, the two activity bands are not agglomerates of a single subunit, but rather have a multisubunit composition, and that the larger band is most likely not a mere multimer of the lower one. It has at least some unique subunits that are absent from the lower band (Fig. 2; arrows). Owing to the horizontal smear, however, the presence of the B14 subunit in the lower band cannot be ascertained at this point. A cross-reaction of the antibody against the 39 kDa subunit of bovine complex I with the *P. serpens* lysate has been described elsewhere [13]. Using the same antibody, we obtained a strong signal, suggesting a specific reaction with the 39 kDa homolog in *P. serpens* (Fig. 5A). Immunodetection of the target complex I subunit in the two-dimensional gel clearly revealed its presence in both the large (~ 2.2 MDa) and small (~ 0.7 MDa) forms of the NADH dehydrogenase (Fig. 5B,C), confirming that these forms share subunits.

Finally, we studied the direct inhibitory effects of piericidin, rotenone and DPI on the NADH dehydrogenase activities in the mitochondrial lysate of *P. serpens*, using ubiquinone 10 and ferricyanide as electron acceptors. Measurable NADH:ubiquinone 10 oxidoreductase activity was about two times lower than the NADH:ferricyanide electron transfer (Table 1). Whereas 100 μM DPI inhibits about 50% of the NADH dehydrogenase activity regardless of the electron acceptor, both 5 μM piericidin and 10 μM rotenone inhibit almost half of the NADH:ubiquinone 10 oxidoreductase activity, but have essentially no effect on the NADH:ferricyanide activity (Table 1). When 2 μM rotenone was added to the lysate, the measured effect was very similar to that obtained with 10 μM of the drug,

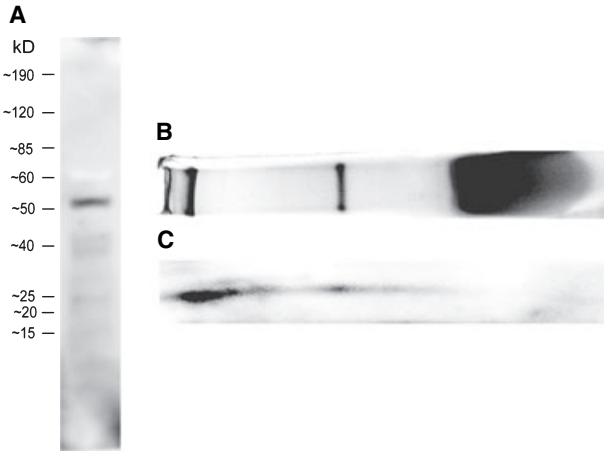

Fig. 5. Immunodetection in a 10%Tricine-SDS gel of the 39 kDa subunit of eukaryotic complex I in the mitochondrial lysate of *P. serpens* (A). The position of molecular mass markers is indicated on the left. The immunopositive signal in a 2D 2–15% BN/10% Tricine-SDS gel (C) comigrates with the in-gel activity staining in a 2–15% BN gel (B).

Table 1. Specific NADH dehydrogenase activity of *P. serpens* and its inhibition. NADH dehydrogenase activity was measured in the mitochondrial lysates of *P. serpens* as described in Experimental procedures. Two different electron acceptors and three inhibitors were used. Medium values of 4–10 experiments, SD and average percentages of inhibition are shown. One unit (U) of activity catalyzes the oxidation of 1 nmol NADH·min^{-1}. Specific activity is calculated as U·(mg mitochondrial protein)$^{-1}$.

Electron acceptor	Inhibitor	Specific activity (U·mg^{-1})	Average inhibition (%)
Ubiquinone 10	–	30 ± 10	0
	5 μM Piericidin	16 ± 4	48
	10 μM Rotenone	20 ± 3	35
	100 μM DPI	18 ± 2	41
Ferricyanide	–	60 ± 17	0
	5 μM Piericidin	54 ± 18	9
	10 μM Rotenone	52 ± 12	11
	100 μM DPI	31 ± 5	49

whereas a concentration of 50 μM appeared to block all NADH dehydrogenase activities (data not shown).

Discussion

Despite the fact that some strains of *Phytomonas* represent economically important pathogens of cassava, coffee plants, and coconut and oil palms, almost nothing is known about the mitochondrial functions of these flagellates other than that they lack respiratory complexes III and IV [31,32]. However, this important feature represents a practical advantage for studying complex I in this trypanosomatid. The mitochondrial membrane of *Phytomonas* is likely to have a less complex protein content, and no interference of complexes III and IV with activity measurements can occur.

Alternative NADH dehydrogenase mimics the activity of complex I [23,24,33], and previous attempts to chromatographically separate these activities in *P. serpens* were not convincing [13]. Therefore, we resorted to another approach. As complex I has previously been detected in the mitochondrial lysates of *Try. brucei* and *P. serpens* by in-gel activity staining [14], we decided to further explore this approach by combining it with gradient BN/Tricine-SDS gel electrophoresis complemented with inhibition experiments. This approach allowed the detection of an activity band migrating at ~2.2 MDa in *P. serpens*, along with a previously described lower band with mobility similar to that of the putative complex I of procyclic *Try. brucei* [14]. Resolution of the BN gel in the second dimension revealed a multisubunit composition of the complexes in question. Whereas both small and large complexes shared several subunits, the large form seems to contain a number of unique subunits. One of the subunits found in both putative forms of *P. serpens* complex I is most likely a homolog of the 39 kDa subunit of bovine complex I.

From all the sequenced subunits derived from the activity band, only one protein showed an unambiguous hit in the databases, namely with the B14 subunit of the mammalian NADH dehydrogenase. Being much larger than its homologs in other organisms, the B14 subunit of trypanosomatids could reflect substantial differences between complex I subunits in these primitive eukaryotes and those outside of the Kinetoplastida. Such a divergence occurred despite the fact that this subunit is considered to be an ancestral eukaryotic core subunit, situated at the basis of the peripheral arm within subcomplex Iα [25]. This occurrence is not without precedent, as highly divergent or even unique subunits constitute other respiratory complexes in trypanosomatids [34]. Finding this subunit together with a homolog of the 39 kDa subunit further supports the notion that in *P. serpens* the detected NADH dehydrogenase is a genuine complex I, a conclusion that was indirectly supported by rotenone and cross-reacting antibodies [13,35]. So far, in the studied flagellate, we have no direct evidence for the predicted ability of complex I to shuttle electrons across the mitochondrial membrane. However, the lack of sensitivity of membrane potential to the inhibitors of complex V and its sensitivity to rotenone [35] strongly point towards such a role for complex I, which is the only other complex that can uphold potential in the mitochondrion of this flagellate [32].

Each of the drugs inhibited between 35% and 48% of total electron flow from the NADH dehydrogenase complex I to ubiquinone 10 in the *P. serpens* mitochondrial lysate. As rotenone and piericidin are specific inhibitors of complex I in eukaryotes, we assume that they bind to the large form of the complex, which contains a homolog of the B14 subunit of mammalian complex I. The residual activity is most likely provided by the alternative NADH dehydrogenase, which is known to be resistant to both inhibitors [23,24,33]. Moreover, the putative small form of complex I (subcomplex I) may be insensitive to these drugs, as described for bovine complex I [36]. It appears that two sources contribute 40% of the total NADH dehydrogenase activity inhibited by DPI. On the basis of the in-gel inhibition experiments, we propose that this drug inhibits both the alternative NADH dehydrogenase, a well-documented target of DPI [24,29], as also confirmed by our results with *Y. lipolytica*, and the putative subcomplex I. The discriminatory effect of DPI excluded the possibility that the ~2.2 MDa large complex is just an oligomer of the small form. The different influences of DPI on the two forms could be caused by its failure to access the flavin cofactor in the large form of complex I, whereas the cofactor remains accessible in the small form. Indeed, to address this possibility, experiments with prolonged preincubation time have been performed, in which the partial DPI inhibition was also observed in the large form (data not shown). However, the unlikely possibility that the large form is in fact an NADH dehydrogenase lacking any flavins cannot be excluded at this point.

The existence of two forms of complex I in *P. serpens* could be a consequence of a partial split of complex I during the isolation procedure into its membrane-bound part and peripheral arm. This possibility is supported by the fact that the relative intensity of the lower band varies with the conditions under which the mitochondrial lysate has been prepared (data not shown). In a less likely scenario, both bands with NADH dehydrogenase activity correspond to complexes that differ in their subunit composition, coexist in *P. serpens*, and have different functions.

Experimental procedures

Cultivation and isolation of mitochondria from *Y. lipolytica*

The *Y. lipolytica* strain E129 (*MatA lys11-33 ura3-302 leu2-270 xpr2-322*) was grown in YPD medium consisting of 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose for 24 h at 28 °C. The cells were harvested by centrifugation (1000 ***g***, 5 min, 4 °C, U-32R centrifuge; Boeco, Hamburg, Germany; rotor type 1617) and washed twice with cold, sterile water. The pellet was washed in 2 mL of 1.2 M D-sorbitol, and resuspended in 10 mL of solution A [0.5 M D-sorbitol, 10 mM EDTA, pH 7.0, 50 mM Tris/HCl, pH 7.4, 2% (v/v) 2-mercaptoethanol, 1 $mg \cdot mL^{-1}$ zymolyase]. The mixture was gently shaken for 45 min at 37 °C, and briefly vortexed before centrifugation (250 ***g***, 10 min, 4 °C, U-32R centrifuge, rotor type 1617). The pellet was resuspended in 10 mL of mito-washing buffer (0.5 M D-sorbitol, 1 mM EDTA, pH 7.0, 50 mM Tris/HCl, pH 7.4), vortexed again, and spun (250 ***g***, 10 min, 4 °C, U-32R centrifuge, rotor type 1617). Finally, the supernatant was centrifuged (16 000 ***g***, 10 min, 4 °C, U-32R, rotor type 1689L), and the mitochondrial pellet was stored at − 80 °C.

Cultivation and isolation of kinetoplasts

P. serpens strain 1G, originally isolated from its insect vector, was cultured in brain heart infusion medium with 10 $\mu g \cdot mL^{-1}$ hemin at 26 °C. The kinetoplast–mitochondrial vesicles from 5×10^8 cells were isolated by hypotonic lysis as described elsewhere [14]. Pelleted mitochondrial vesicles were stored at − 80 °C until further use.

NADH dehydrogenase activity assays

Kinetoplast–mitochondrial vesicles isolated from 5×10^8 cells were resuspended in 40 μL of 1 M aminocaproic acid, and the addition of 10 μL of 10% dodecylmaltoside was followed by 1 h of incubation at 4 °C. The lysate was spun in a microcentrifuge (15 600 ***g***, 10 min, 4 °C, 5414 centrifuge; Eppendorf, Hamburg, Germany; 12-place fixed angle rotor), and the protein concentration was determined by the Bradford assay. Next, the supernatant was used to determine the NADH dehydrogenase activity using two artificial electron acceptors: ubiquinone 10 and ferricyanide. The NADH:ubiquinone 10 and NADH:ferricyanide oxidoreductase activities were measured in a 1 mL cuvette containing NDH buffer (50 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 0.2 mM KCN), 5 μL of mitochondrial lysate, and 5 μL of 20 mM NADH. After addition of 10 μL of 2 mM oxidized ubiquinone 10 or 5 mM ferricyanide, the change in absorbance at 340 nm was measured every 10 s for 3 min. A unit of activity was defined as the amount of enzyme that catalyzes the oxidation of 1 nmol $NADH \cdot min^{-1}$, assuming an extinction coefficient of 6.2 $mM^{-1} \cdot cm^{-1}$ [13]. Solutions of the inhibitors were freshly prepared. Piericidin was dissolved in ethanol, rotenone in dimethylsulfoxide, and DPI in methanol. Inhibitors were added to the assay mixture immediately before the start of the reaction. Different concentrations of rotenone were used, as indicated in Results.

In-gel activity staining

One hundred micrograms of proteins from the mitochondrial lysate was mixed with 1.5 μL of CB solution [0.5 M aminocaproic acid, 5% (w/v) Coomassie Brilliant Blue (CBB) G-250], incubated for 10 min on ice, and run on 2–15% gradient BN gel. For the in-gel activity staining of complex I, the gel was transferred to reaction buffer (0.1 M Tris/HCl, pH 7.4, 0.14 mM NADH, 1 $mg{\cdot}mL^{-1}$ nitrotetrazolium blue chloride) immediately after the run and stained by slow agitation overnight. In the case of inhibition, the gel was incubated in reaction buffer with 100 μM DPI. The enzymatic activity of complex I appears as a specific violet precipitate. The gel was subsequently fixed in a mixture of 30% methanol and 10% acetic acid [37].

Two-dimensional gel electrophoresis and western blot analysis

Analysis of respiratory complexes of purified mitochondria was performed with two-dimensional BN/Tricine-SDS gel. One hundred micrograms of mitochondrial lysate prepared as described above was loaded per lane, analyzed on 2–15% gradient BN gel, and resolved in 10% Tricine-SDS gel. After electrophoresis, the gel was stained with CBB. The mitochondrial lysate was resolved in two-dimensional BN/Tricine-SDS gel, blotted, and probed with a monoclonal antibody raised against the 39 kDa subunit of bovine NADH:ubiquinone oxidoreductase (1 : 250) (Molecular Probes, Eugene, OR, USA) and secondary anti-mouse serum (1 : 1000) (Sevapharma, Prague, Czech Republic). Secondary antibodies coupled to horseradish peroxidase were visualized according to the manufacturer's protocol using the ECL plus kit (Amersham Biosciences, Chalfont St Giles, UK).

In-gel digestion and MS

CBB-stained spots were excised from the gel and subjected to reduction by 20 mM Tris(2-carboxyethyl)phosphine in 50 mM Tris/HCl (pH 8.1) at 75 °C for 30 min. Reduced cysteines were alkylated by incubation with 30 mM iodoacetamide at 37 °C for 40 min. Next, the gel pieces were washed, dried in a SpeedVac concentrator (Savent Instruments, Holbrook, NY, USA), and rehydrated with a solution of 50 mM ethylmorpholine acetate (pH 8.2), 10% acetonitrile, and 0.1 $\mu g{\cdot}\mu L^{-1}$ trypsin (Roche, Mannheim, Germany). Digestion was carried out overnight at 37 °C. Peptides were extracted from the gel and analyzed by LC-MS/MS. The tryptic peptides were loaded onto a capillary column (0.10 × 100 mm) packed with 10 cm of reversed phase resin (MAGIC C-18, 200 Å, 5 μm; Michrom BioResources, Auburn, CA, USA) and resolved using a gradient from 5% acetonitrile/0.5% acetic acid to 35% acetonitrile/0.5% HOAc over 50 min. The eluting peptides were directly analyzed with an ion trap mass spectrometer (LCQ^{DECA}; ThermoQuest, San Jose, CA, USA). Full-scan spectra were recorded in positive mode over the mass range 350–1800 a.m.u. MS/MS data were automatically acquired on the two most intense precursor ions in each full-scan spectrum. Tandem mass spectra were interpreted manually and with SEQUEST software. For searches, the 'no protease' option was chosen, and potential (oxidation of Met) and static (alkylation of Cys) modifications were enabled. SEQUEST results were processed according to the criteria described elsewhere [38].

Acknowledgements

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