Cryo-slicing Blue Native-Mass Spectrometry (csBN-MS), a Novel Technology for High Resolution Complexome Profiling

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Blue native (BN) gel electrophoresis is a powerful method for protein separation. Combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), it enables large scale identification of protein complexes and their subunits. Current BN-MS approaches, however, are limited in size resolution, comprehensiveness, and quantification. Here, we present a new methodology combining defined sub-millimeter slicing of BN gels by a cryo-microtome with high performance LC-MS/MS and label-free quantification of protein amounts. Application of this cryo-slicing BN-MS approach to mitochondria from rat brain demonstrated a high degree of comprehensiveness, accuracy, and size resolution. The technique provided abundance-mass profiles for 774 mitochondrial proteins, including all canonical subunits of the oxidative respiratory chain assembled into 13 distinct (super-)complexes. Moreover, the data revealed COX7R as a constitutive subunit of distinct super-complexes and identified novel assemblies of voltage-dependent anion channels/porins and TOM proteins. Together, cryo-slicing BN-MS enables quantitative profiling of complexes with resolution close to the limits of native gel electrophoresis. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.054080, 669–681, 2016.

Blue native (BN)1-PAGE and its colorless variant, colorless native PAGE, were originally developed by Schägger and This is an open access article under the CC BY license.

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Received August 11, 2015, and in revised form, November 12, 2015
Published in MCP Papers in Press, November 23, 2015, DOI 10.1074/mcp.M115.054080

Author contributions: T.B. and U.S. designed research; C.S.M., A.H., T.B., C.H., B.F., and U.S. contributed new reagents or analytic tools; A.H., T.B., C.H., B.F., and U.S. performed research; C.S.M., A.H., and T.B. contributed new research tools; W.B., and L.E. performed research; C.S.M., A.H., and T.B. contributed new reagents or analytic tools; A.H., T.B., C.H., B.F., and U.S. performed research; C.S.M., A.H., and T.B. contributed new reagents or analytic tools; W.B., and L.E. performed research; A.H., T.B., C.H., B.F., and U.S. contributed new reagents or analytic tools; and C.S.M., A.H., and T.B. analyzed data; B.F. and U.S. wrote the paper.

1 The abbreviations used are: BN, blue native; 2D, two-dimensional; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; csBN-MS, cryo(-microtome) slicing BN-MS; dd, double-deionized (Milli-Q); FT, Fourier transform; FWHM, full width at half-maximum; HA, (influenza) hemagglutinin; IB, isolation buffer; OXPHOS, oxidative phosphorylation; PV, peak volume; TEMED, tetramethylethylenediamine; TOM, translocase of the mitochondrial outer membrane; VDAC, voltage-dependent anion channel.
teins. In these studies, 82/73 (including 8 single-peptide hits) and 55/54 (including 7 single-peptide hits) of the 90 known OXPHOS complex subunits were identified/clustered, respectively. Furthermore, TMEM126B was identified as a novel and essential subunit of an OXPHOS complex I assembly complex (13).

Notably, all of these studies achieved clustering of protein profiles for the dominating populations of complexes, although they largely failed to obtain information on sub-complexes and super-complexes, most likely as a consequence of the strong undersampling in the first dimension (well below the resolution of BN-PAGE) and a limited dynamic range of MS-based identification and quantification.

To improve the resolution of BN-MS for analysis of protein super-complexes and their subunit composition, we have recently started to develop sub-millimeter sampling of BN gel lane sections by using cryo-microtome slicing (15, 16). Here, we describe a new methodology for comprehensive and high resolution complexome profiling that combines this high resolution gel sampling method with a sensitive and precise label-free MS quantification workflow. Protein profiles determined in a mammalian mitochondrial membrane preparation showed a highly effective mass resolution (<5% molecular weight difference) over the whole BN-PAGE separation range and together covered a major portion of the mitochondrial membrane proteome.

**EXPERIMENTAL PROCEDURES**

**Mitochondrial Membrane Preparation**—Mitochondria were isolated from freshly dissected adult rat brains following the procedure by Rendon and Masmoudi (17). 15 g of brain tissue were washed in isolation buffer (IB, 320 mM sucrose, 6 mM Tris-HCl, pH 7.5, 6 mM EDTA + protease inhibitors) and gently homogenized with 70 ml of IB using a glass potter. An equal volume of IB was added, and the homogenate was centrifuged for 5 min at 1,100 × g. Pellets were re-homogenized by repeating this procedure, and the combined supernatants were pelleted for 10 min at 17,000 × g, resuspended in IB, and pelleted again for 20 min. The washed pellet was homogenized in 30 ml of IB, loaded onto Ficoll step gradients (9 ml on 7 ml of 13% Ficoll in IB), and centrifuged at 100,000 × g for 30 min. The fractions at the 7.5/13% Ficoll interphase containing vesicle-enclosed mitochondria were collected, diluted with IB, and pelleted (10 min at 18,000 × g).

These vesicles were lysed in 6 mM Tris-HCl, pH 8.1, and protease inhibitors (40 ml, 30 min), and the membranes were collected by ultracentrifugation (15 min at 75,000 × g). After resuspension in 3 ml of IB, the membranes were separated on an isosotic step gradient (1 ml/gradient) built by 10%, 20%, and 40% Percoll (GE Healthcare) in IB by centrifugation for 20 min at 37,000 × g. Mitochondria were retrieved from the 20–40% Percoll interphase, washed twice in IB by dilution/centrifugation/resuspension cycles, and stored as a concentrated suspension (56 mg/ml) at −80 °C.

**Preparative BN-PAGE**—Non-denaturing 1–13% (v/v acrylamide) gradient gels (14 × 11 cm, 1.5-mm spacer) were cast using a periodic pump fed by a linear gradient mixer filled with two solutions with freshly added ammonium persulfate and TEMED as follows: solution A (13 ml), 11.5% acrylamide, 0.45% bisacrylamide, 0.75 M aminocaproic acid, 50 mM BisTris, pH 7.0, 10% glycerol; and solution B (13 ml), 0.95% acrylamide, 0.025% bisacrylamide, 0.75 M aminocaproic acid, 50 mM BisTris, pH 7.0, 0.2% ComplexIolyte 47 detergent (Logopharm, Germany), and polymerized overnight. Gels were run in water-cooled (10 °C) Penguin M electrophoresis systems (PEQLAB, Germany) using the cathode and anode buffer system as described previously (2). 1 mg of mitochondrial membranes was solubilized in 0.8 ml of detergent buffer (1% ComplexIolyte 47 (protein-detergent ratio of 1:8) with salts replaced by 0.5 mM aminocaproic acid, 50 mM imidazole, pH 7.0, 1 mM EDTA, 1 mM EGTA, and protease inhibitors) for 30 min on ice and cleared by ultracentrifugation (11 min at 79,000 × g, S80-AT3 rotor with tube adaptors). The solubilize was supplemented with 10% glycerol, 0.1% Coomassie G-250 and directly loaded onto the gel (20 μg/mm2 gel cross-section). Voltage was initially set to 100 V for 30 min, ramped to 500 V during 1 h, and kept at 500 V for 8 h.

**Sample Preparation**—After BN-PAGE separation, gel lanes were excised (5-mm strip was dissequered for 2D analysis, Fig. 1A) and frozen at −20 °C. For 2D-BN-SDS-PAGE, the narrow strip was incubated twice for 5 min in 5 ml of 2× Laemmli buffer and placed on a 13% SDS-polyacrylamide gel silver-stained after the run. The broad gel strip was fixed twice (for 5 min in 30% ethanol, 15% acetic acid), washed briefly in ddH2O, and equilibrated in tissue embedding solution (Leica Tissue Freezing Medium, WWR, Germany) for 20 min under gentle movement. The equilibrated gel strip was cut into three sections (Fig. 1A, colored boxes), and each of them was embedded for cryo-microtome slicing as illustrated in Fig. 2. Gel pieces were carefully placed on a frozen block of embedding solution in casting molds (3 × 3 cm, half-filled) with the protein migration front perfectly aligned with one of the mold walls to avoid any distortion of the gel. The gel position was fixed with needles before overlaying it with embedding solution and freezing. The frozen blocks were mounted on precooled probe supports and equilibrated to a temperature of −19 °C in the cryo-microtome (Leica CM1950, Germany). Slicing was done by slow manual driving of the specimen feed set to a step size of 0.3 mm (angle of 0°). Around 80 successive slices from each block were separately collected and stored frozen. Prior to digestion, the embedding medium was largely removed by washing the gel strips twice in 30% ethanol, 15% acetic acid and ddH2O, once in 100% ethanol and again twice in ddH2O. In-gel digestion was carried out according to the procedure described previously (18) using sequencing grade modified trypsin (Promega, Germany; 1:200 in 25 mM NH4HCO3).

**LC-MS/MS Analysis**—Extracted vacuum-dried peptides were redissolved in 20 μl of 0.5% trifluoroacetic acid for each of the 230 samples and transferred into individual well plate vials. Using an UltiMate 3000 RSLCnano ( Dionex/Thermo Scientific, Germany), 5 μl of each sample were trapped on a C18 PepMap100 precolumn (particle size 5 μm; Dionex/Thermo Scientific) with eluent A (5 min, 20 μl/min) and separated in a PicoTip™ Emitter (inner diameter 75 μm, tip 8 μm; New Objective) manually packed with ReproSil-Pur 120 ODS-3 (C18; particle size 3 μm; Dr. Maisch HPLC, Germany) with an aqueous-organic gradient (eluent A: 0.5% (v/v) acetic acid; eluent B: 0.5% (v/v) acetic acid in 80% (v/v) acetonitrile; nano/cap pump gradient: 5 min 3% B, 60 min from 3% B to 30% B, 15 min from 30% B to 99% B, 5 min 99% B, 5 min from 99% B to 3% B, 15 min 3% B; flow rate 300 nL/min). Eluting peptides were electrosprayed (2.3 kV; transfer capillary temperature 250 °C) in positive ion mode into an Orbitrap Elite tandem mass spectrometer equipped with a Nanospray Flex Ion Source (Thermo Scientific; total acquisition time 105 min according to the gradient). FT full MS (m/z 370 to 1,700; resolution 240,000; flex waveforms) and data-dependent ion trap MS/MS spectra (maximum of 10 per scan cycle; normal scan rate; centroid data) were acquired with target values of 1,000,000 and 10,000, and maximum injection times of 500 and 200 ms, respectively. Injection waveforms were enabled for all spectra. The following data-dependent settings were used: dynamic exclusion enabled (repeat count, 1;
exclusion list size, 500; repeat/exclusion duration, 30 s; exclusion mass width, ±20 ppm); ion trap MS/MS ion injection time predicted; preview mode for FT-MS master scans, charge state screening, mono-isotopic precursor selection, and charge state rejection (+1) enabled; minimum signal threshold, 500 counts; activation type, collision-induced dissociation (default charge state, 2; isolation width, 2.0 m/z; normalized collision energy, 35; activation Q, 0.25; activation time, 10 ms).

Protein Identification—Peak lists were extracted from fragment ion spectra using the mconvert.exe tool (part of ProteoWizard, version 2.2.3214; default parameters). For each dataset, all precursor m/z values were shifted by the median m/z offset of all peptides assigned to proteins in a preliminary database search with 15 ppm peptide mass tolerance. Corrected peak lists were searched with Mascot 2.5.1 (Matrix Science, UK) against the UniProtKB/Swiss-Prot database (release 2015_02; only P00761, P00766, and P02769 and all rat, mouse, and human entries) supplemented with 349 rat UniProtKB/TrEMBL or NCBI RefSeq entries (identified by BLAST searches of mitochondrial proteins for which no rat homologs existed in the UniProtKB/Swiss-Prot database). Acetyl (protein N terminus), carbamidomethyl (Cys), Gin > pyro-Glu (N-terminal Glu), Glu > pyro-Glu (N-terminal Glu), oxidation (Met), and propionamide (Cys) were chosen as variable modifications; peptide, and fragment mass tolerance were set to ± 5 ppm and ± 0.8 Da, respectively, and one missed tryptic cleavage was allowed. The expectation value cutoff for peptide identification was set to 0.5. Related identified proteins (subset or species homologs) were grouped together using the name of the predominant member.

The final list of identified mitochondrial proteins (supplemental table 1) was obtained after filtering the search results obtained for the 230 samples for proteins that were (i) localized or linked to mitochondria according to UniProtKB/Swiss-Prot database annotation or to the MitoCarta database (entries manually updated to the current UniProtKB/Swiss-Prot protein information), and (ii) whose identification was based on at least two independent protein-specific peptides (at least one with maximum expectation value < 0.05) found in at least two slice samples. An exception, proteins were accepted with only one identified specific peptide (with maximum expectation value < 0.05 and in at least two slice samples) when at the same time more than 10% of the detectable tryptic peptides of that protein were identified (see supplemental table 1). The raw data and database search results (mzIdentML format) have been submitted to the PRIDE repository (project accession code PXD002681).

Protein Quantification—Label-free quantification of proteins was carried out following the principles described previously (19) with further optimizations to address the specific challenges arising from combining 230 complex datasets. Peptide signal intensities were integrated over time and m/z as peak volumes (PVs) from FT full scans using MaxQuant version 1.4.12.1 with integrated off-line mass calibration (20). For correcting peptide PV elution time shifts, individual datasets were aligned one-by-one to reference peptide elution times using LOESS regression. These reference times were dynamically calculated from the median peptide elution times over all previously aligned datasets and updated accordingly after each alignment. The process started with the largest dataset as seed and successively aligned the dataset having the highest overlap with the reference peptide list. PVs were then assigned to peptides based on their m/z and elution time, values that were obtained either directly from MS/MS-based identification or indirectly, i.e., from identifications in parallel datasets (termed “inserted”). Alignment and assignment were carried out by software developed in-house using parameters that resulted in effective m/z and elution time matching tolerances of ±2.5 ppm and ±1 min, respectively. The assigned PV data has been formatted as mzQuantML file and submitted to the PRIDE repository (project accession code PXD002681). To correct for run-to-run variations in peptide recovery and ionization efficiency of the LC-MS setup, PVs of each dataset n were rescaled by the median of all peptide scale factors s with s = C \text{max, PV}_n / \text{PV}_n (see supplemental figure 1). The PV data for each protein were filtered for outliers and false-positive assignments using a new correlation-based method. For each protein and over all 230 datasets, each PV was checked for its consistency with other PVs of the same protein (lines and columns in supplemental table 2) by finding all 2-by-2 sub-matrices containing the respective PV and calculating their scores s = (A/B)/(C/D) (i.e., the ratios of the pairs of connected PV ratios). The consistency of each PV was then determined from the sum of all of its associated sub-matrix scores s after reciprocal normalisation (s = 1/s if s > 1) and weighting (with the sum of the three other matrix elements (PVs)); very consistent PV values with a sum score <0.2 were finally eliminated.

PVs of each peptide were then normalized to their maximum over the 230 datasets to obtain relative peptide profiles. These were subsequently ranked for each protein by pairwise Pearson correlation (19). The average of at least two (one for the exception of proteins identified by one specific peptide (see under “Protein Identification”)) and up to 6 or 50% (whichever value is greater) best correlating protein-specific peptide profiles over a window of three consecutive slices was used to calculate the relative abundance values of each protein present in the respective profiles. Finally, these protein profiles were least squares-fitted to the normalized (molecular) abundance (abundancenorm) values (19) determined for the respective protein in the 230 datasets as a rough measure of molecular abundance (supplemental table 3).

Characterization of Protein Complexes—Individual protein profiles were analyzed by finding peaks in abundance mass profiles (up to three per protein) using continuous wavelet transformation (Mexican Hat) and fitting a Gaussian function to these peaks (Mathematica, Wolfram Research, version 9.0.1) and Python/SciPy (versions 2.7.6 and 0.15.1). Thus, each protein or protein complex was characterized by the position of its abundance peak maximum and by the FWHM value (both parameters were given in slice number units). The smallest FWHM values represented a measure for the effective size resolution of the approach (supplemental table 4). The slice numbers were converted to apparent molecular sizes (apparent mass in kDa) using the result of the linear regression of the slice number positions of 31 reference protein complex peaks versus their log$_2$($M_r$) values (as reported in UniProtKB/Swiss-Prot, see supplemental table 5). The relative abundance-mass profiles of OXPLOS complexes I–V were determined by the median of the relative abundance profiles of the respective core complex protein subunits (supplemental table 6). For identification of novel protein complexes, defined complex assemblies (supplemental table 4) were screened against the profiles of all proteins determined by the csBN-MS approach using Pearson correlation; the respective results were verified by manual inspection. In addition, single linkage hierarchical clustering of the individual protein abundance profiles was performed with Pearson correlation as distance metric using Python/SciPy (supplemental figure 2).

Biochemical Interaction Analysis in Yeast—The Saccharomyces cerevisiae strain Tom22His and the corresponding wild-type strain YPH499 were described before (21). The yeast strain expressing Por1HA in the YPH499 background was generated by chromosomal integration of a coding region for a triple HA tag and a HIS3 cassette in front of the stop codon of POR1 by homologous recombination (22). Yeast cells were grown on yeast extract/peptone/glycerol medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 3% (v/v) glycerol) at 24–30 ℃. Mitochondria were isolated by differential centrifugation as described (23). For purification of Por1-containing complexes, Por1HA mitochondria were lysed under native conditions with digitonin buffer (0.3% (w/v) digitonin, 10% (v/v) glycerol, 50 mM NaCl, 50 mM Tris-HCl, pH 7.5) and loaded onto a DIY gel filtration column (1 cm × 10 cm) equilibrated with digitonin buffer. The column was eluted with digitonin buffer, and fractions containing the POR1 complex were analyzed by SDS-PAGE.
0.1 mM EDTA, 20 mM Tris/HCl pH 7.4) at a protein/detergent ratio of 1:3 (1 mg of protein/ml) for 15 min on ice. After removal of insoluble material by centrifugation (10 min, 20,000 × g, 4 °C), the samples were subjected to affinity purification via anti-HA chromatography (Roche Diagnostics, Switzerland). After excessive washing with digitonin buffer containing 0.1% (w/v) digitonin, bound proteins were eluted under native conditions by incubation with 1 mg/ml HA peptides (Roche Diagnostics). Tom22His-containing protein complexes were purified by nickel in complex with nitrilotriacetic acid-agarose (Qiagen, Germany) (22). Denatured proteins were analyzed by SDS-PAGE, and native samples were subjected to BN-PAGE. Proteins were transferred by Western blotting onto PVDF membrane and immunodetected using the indicated antisera. To analyze precursor binding to the TOM complex, 35S-labeled Oxa1 was synthesized in a cell-free translation system based on reticulocyte lysate (22). The 35S-labeled Oxa1 precursor was arrested at the TOM complex in the presence of a membrane potential (24). Subsequently, the samples were subjected to affinity purification utilizing the HA tag of Por1 followed by native elution as described above. The 35S-labeled Oxa1 precursor was detected by BN-PAGE and autoradiography.

**Experimental Design and Statistical Rationale**—The mitochondrial membrane preparation (obtained from a pool of eight rat brains to eliminate biological variations) was solubilized and resolved by preparative BN-PAGE three times. The gel offering the most homogenous and sharpest separation of protein bands was processed for csBN-MS analysis. Each gel slice was analyzed in a single LC-MS run. Because of the large proteomic overlap of neighboring slices, subsequent evaluation was designed to use information from consecutive slices to largely eliminate LC-MS intensity variations and to corroborate protein quantification. As a result, each data point in the obtained 774 protein profiles was on average based on 13 PV values (allowing for calculation of robust means). Biochemical experiments in Fig. 6 were repeated at least two times (Fig. 6B: n = 4, Fig. 6C: n = 2–5).

**RESULTS**

**Outline and Workflow of csBN-MS**—For a comparative benchmark application of the csBN-MS approach, we used the proteins and protein complexes present in a membrane preparation of mitochondria isolated from rat brain (see also “Experimental Procedures” and Fig. 1). This protein preparation was well separated on native gels when loaded under conditions optimized for solubilization and total amount of protein load. Fig. 1A illustrates the preparative BN-PAGE separation of 1 mg of protein using a mild detergent condition (in 0.8 ml of ComplexioLyte 47) on a 1–13% acrylamide gradient gel of about 8 cm length. Both the BN gel and the silver-stained second dimension SDS-polyacrylamide gel displayed the well focused band or spot patterns typical for mitochondrial OXPHOS and other (super-)complexes that were distributed over an estimated molecular mass range of 100–3000 kDa (Fig. 1A). In addition, more detailed inspection of the stained 2D gel revealed several sharply focused complexes such as the propionyl-CoA-carboxylase (consisting of subunits PCCA and PCCB), the signals of which extended over gel ranges as small as 1 mm (inset of Fig. 1A).

These results emphasize that quantitative profiling of protein complexes exploiting the full separation capacity of native gels requires (i) gel sampling in the sub-millimeter range together with (ii) high precision quantitative LC-MS/MS analyses. These challenges promoted development of the csBN-MS approach whose workflow and key characteristics are outlined in Fig. 1, B and C. Accordingly, sample preparation is performed by gel slicing with a cryo-microtome that operates at defined step sizes between 0.1 and 0.5 mm and thus enables collection of a large set of uniform gel samples (Fig. 1B) (15, 16). For the aforementioned mitochondrial membrane preparation, cryo-slicing provided a total of 230 slices, each covering 0.3 mm of the BN gel. These gel slice samples were subsequently subjected to high performance LC-MS/MS analyses on an Orbitrap Elite tandem mass spectrometer (see “Experimental Procedures”), and the data were evaluated using procedures optimized for label-free quantification of proteins over large sets of MS data (Fig. 1C). In the entire set of cryo-slices, these analyses unambiguously identified more than 1,200 proteins, 774 of which were bona fide mitochondrial proteins (supplemental table 1). Performance of MS sequencing may be exemplified for the PCCA/PCCB subunits of the propionyl-CoA-carboxylase (Fig. 1A, inset, lower panel). For label-free quantification, the high resolution (240,000 FWHM) LC-MS data were extracted and mass-calibrated offline by MaxQuant providing integrated peak volumes (PVs) (20). These were further processed using software developed in-house for alignment of elution times, compensation of signal intensity variation between runs, and filtering of peptide PV outliers based on their internal consistency. From these data (supplemental table 2) peptide and protein profiles were finally determined and used for correlation analysis to identify complexes and to determine their composition and molecular size (Fig. 1C). The key procedures of the csBN-MS analysis are described in more detail under “Experimental Procedures” and below.

**Handling of BN Gels and Cryo-slicing**—High resolution slicing of broad (2 cm) BN gel strips is a key element of csBN-MS that requires precise handling with respect to both geometry and positioning of the gel strips throughout the whole procedure illustrated in Fig. 2.

In a first step, the gel strip must be thoroughly equilibrated in the viscous embedding medium and relieved from mechanical stress before it can be trimmed into sections (boxed areas in Fig. 1A) to accommodate the operating range of the cryo-microtome (Fig. 2A). These sections are subsequently placed on a plain frozen block of embedding medium prepared in a casting mold (Fig. 2B), transiently fixed in their position with needles, overlaid with embedding medium, and frozen at −20 °C. In the next step, the frozen blocks are mounted on the cryo-microtome probe support, equilibrated to a temperature of −19 °C, and slowly cut with a steel blade (manually driven at 1 cycle per 5 s; Fig. 2C). Finally, the frozen gel slices were individually collected, and the embedding medium was removed (Fig. 2D). After re-thawing and rinsing, the gel slices usually exhibit uniform size and thickness and were sub-
jected to tryptic in-gel digestion after additional washing steps to avoid critical background and/or column clogging during LC-MS/MS analysis due to residual embedding medium constituents.

Processing of MS Data Obtained from Large Sets of BN Cryo-slices—Despite their small size, each of the 230 cryo-slices from the mitochondrial membrane fraction represented a complex protein mixture containing an average of 391 identified proteins or 2,745 peptides, respectively. Therefore, all steps of quantitative complexome analysis (Figs. 3 and 4), including proper signal assignment to individual proteins over the extended set of slice samples as well as label-free quantification of protein amounts, critically depend on the quality of the primary LC-MS data. The histograms in Fig. 3A illustrate the distributions of relative mass errors after off-line calibration (left) and deviations in elution time after time alignment (right) of all LC-MS signals (PVs) either identified by MS/MS sequencing or assigned indirectly based on m/z and elution time (referred to as “sequenced” and “inserted” in Fig. 3A).

Noteworthy, both distributions are bell-shaped and symmetrical and lack obvious offsets, indicating a low rate of assignment errors and a narrow effective window for both mass and time (around 2.5 ppm and 1 min, respectively).

The MS signals found for the identified mitochondrial proteins were used to generate PV-based profiles across the entire set of slice samples, a multistep procedure that is exemplified in Fig. 3B for cytochrome c oxidase subunit NDUFA4 (NDUA4), a protein of intermediate abundance. The
PVs initially assigned to the 20 identified peptides of NDUA4 spanned around 3–4 orders of magnitude and exhibited considerable variation in intensity, discontinuities, and gaps over the set of slice samples (Fig. 3B, 1st panel). In the first step, these PVs were rated by their internal consistency (see “Experimental Procedures”), and the 20% lowest scoring PV values were removed. Next, the global slice-to-slice variations caused by intrinsic fluctuations of peptide recovery and ionization efficiency were eliminated. As for the vast majority of proteins, abundance values were expected to remain unchanged in the neighboring slices; this could be readily done by using the deviation of the PV median (in a given slice) from the local average median PV for re-scaling (factors are indicated in supplemental figure 1). Both measures led to a significant reduction of the noise in the PV profiles (Fig. 3B, 2nd panel). The filtered and re-scaled PVs for each peptide were then normalized to their maximum to yield relative peptide abundance profiles. Subsequently, these profiles were ranked by pairwise linear correlation (19), and the 50% most consistent peptide profiles were selected (Fig. 3B, 3rd panel). Finally, the relative protein abundance value in each slice was calculated from the average of the selected normalized PV values over a window of three slices, and after least square fitting to the respective abundance,corr, values (19), the molecular abundance profile of NDUA4 was determined (Fig. 3B, 4th panel). Application of this procedure to the whole PV dataset of the mitochondrial protein preparation established 774 highly resolved protein profiles (supplemental table 3).

These protein profiles were next used to estimate the effective mass resolution of the csBN-MS approach and to derive an apparent mass scaling for complexes on the BN gel. With respect to mass resolution, we sought to determine the FWHM of protein complex peaks as a measure readily accessible in data such as illustrated in Fig. 4A (gray line). Notably, all these complexes focused within 3–9 slices (corresponding to gel ranges of 1–2.7 mm), values that were essentially constant over the entire gel range investigated and that did not display any systematic difference between soluble proteins (Fig. 4A, upper panel) and those associated with integral membrane proteins (Fig. 4A, lower panel), in line with the notion of Ref. 25 that migrating complexes in BN-PAGE may become largely stripped off their detergent micelles. More detailed (and quantitative) analysis using fits of a Gaussian function to the entire set of protein profile peaks (see “Experimental Procedures” and supplemental table 4) showed that protein peak FWHMs were quite variable among the mitochondrial proteins. A multitude of factors, including complex geometry, molecular heterogeneity, or gel migration artifacts, may contribute to broadening of protein peaks (25). Nevertheless, the best focused protein peaks again suggested a resolution limit of about three slices FWHM (or 1 mm, red line in Fig. 4B) over most of the separation range.

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**Fig. 2.** Embedding and cryo-slicing of BN gels. Documentation of the key steps of sample preparation. A, trimming of gel sections after equilibration. B, their positioning and embedding for freezing. C, mounting and slicing of frozen gel blocks with a cryo-microtome. During all steps, the precise perpendicular orientation of the gel section with respect to the migration front must be maintained. D, left panel, removal of embedding medium from gel slices. Right panel, gel slices after thawing (view on the gel slice cross-section). Slices were numbered 1–230 starting from the low molecular weight end.
**FIG. 3. Assignment of peptide features and determination of protein profiles.** A, *left panel*, histogram showing the distribution of relative mass errors (measured versus theoretical mass) of m/z features assigned to peptides (i) directly through MS/MS fragmentation and Mascot identification ("sequenced," red) or (ii) indirectly based on matching mass, charge, and elution time ("inserted," blue) for all 230 datasets. Note that both distributions are Gaussian-like (mean ± S.D. = 0.008 ± 0.71 ppm (sequenced) and 0.11 ± 0.79 ppm (inserted)). Right panel, histogram displaying deviations from average in elution time determined for m/z peaks across all 230 datasets (same color coding; mean ± S.D. = 0.003 ± 0.13 and 0.01 ± 0.13 min for sequenced and inserted peptide features, respectively). B, processing of peptide PVs for determination of abundance-slice number profiles (detailed under “Experimental Procedures”) exemplified for the mitochondrial protein NDUA4. Primarily assigned peptide PVs (log scale, *panel 1*) were corrected for slice-to-slice variation and filtered for consistency (*panel 2*). PVs of the 50% most consistent peptides (selected by Pearson correlation ranking) were normalized to their maximum resulting in relative peptide abundance profiles (*panel 3*). Relative protein abundances were calculated as averages of the peptide profiles shown in *panel 3* and scaled to the respective protein abundance$_{norm}$ values (19) as an approximate measure of absolute protein abundance (*panel 4*).
A set of 31 narrow-focusing protein complexes was selected for which the UniProtKB/Swiss-Prot database offered information on subunit stoichiometry and theoretical molecular weight (see supplemental table 5). Linear regression of log$_{10}(M_r)$ values versus the slice number position of their peak maxima (see under “Experimental Procedures”) provided a

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**FIG. 4. Effective apparent mass resolution of protein complexes.** A, examples of well focused protein complexes (labeled by their UniProtKB/Swiss-Prot database entry names of representative complex constituents) identified across the entire range of the BN-polyacrylamide gel. Note the similar peak FWHMs obtained for soluble complexes (upper panel) and complexes integral to the membrane (lower panel); the latter included two non-mitochondrial complexes (ITPR1 and RYR2). B, plot of peak FWHM values determined for 635 well defined protein complexes as in A by fits of a Gaussian function to the respective abundance profile maxima. Note the maximum effective resolution of around three slices (1 mm), which was largely independent from the apparent molecular weight of the complexes. C, calibration of apparent molecular size: log$_{10}(M_r)$ values of 31 reference complexes (as reported in the UniProtKB/Swiss-Prot database) were plotted versus the slice number position of the peak amount of the respective complex (circles in gray; supplemental table 5); the calibration function was obtained from these data points by linear regression (red line). Protein complexes in best agreement with the regression line are labeled 1–11 (green).
Application of csBN-MS, Analysis of OXPHOS Complexes and VDAC-TOM Assemblies—The mitochondrial OXPHOS complexes and their higher order assemblies have been extensively investigated and are thought to be assembled from 90 constitutive subunits in mammals (supplemental table 6). Most of these subunits are small hydrophobic proteins that are difficult to analyze by mass spectrometry, but they represent an ideal reference for testing comprehensiveness and sensitivity of the csBN-MS approach.

Fig. 5A illustrates the results obtained for OXPHOS complex V, the mitochondrial membrane ATP synthase that consists of five distinct complexes as follows: the F1 part at a mass of 280 kDa; the dominating monomer and dimeric forms of the intact ATP synthase (600 and 1150 kDa, respectively); and at least two higher molecular weight supercomplexes exhibiting masses of 800 and 1,600 kDa, respectively. Notably, profile peaks for all 11 subunits were detected with approximately the same abundance relations, even for the low abundant complex population at 1,600 kDa (Fig. 5A, inset). An additional small protein, the mitochondrial ATPase inhibitor (ATIF1), was found to closely correlate with the major complex V populations (supplemental table 5), in line with its described role as an endogenous inhibitory interactor of ATP synthase (26).

In contrast to the homogeneous composition of complex V, OXPHOS complex IV (cytochrome c oxidase) displayed a markedly heterogeneous appearance (Fig. 5B). Thus, the high resolution csBN-MS profiles suggest at least four sub-populations of complex IV with distinct subunit composition. This pattern was observed for both the monomeric (mass range of about 250–350 kDa) and the dimeric form of the complex (mass range of about 500–600 kDa).

Finally, Fig. 5C shows the ensemble of OXPHOS complexes I–V resolved by the csBN-MS technique demonstrating their assembly into super-complexes as first demonstrated in the elegant work by Schägger and Pfeiffer (2). At least eight of these supercomplexes were resolved by csBN-MS based on the abundance profiles of complexes I–V (Fig. 5C). Importantly, size resolution and precision of the approach allowed for discrimination of closely overlapping assemblies such as the dimer of complex V (V2) and the complex I–IV supercomplex (I1IV1), the complex I (I1), and the tetrameric complex I–III–IV (I1III1IV1), or the dimeric forms of complexes III (III2) and IV (IV2); the profile peak maxima of all complexes were separated by only 1 or 2 slices (gel range of ~0.5 mm; see also 2D gel in Fig. 1A). In addition, correlation analysis performed with the complex peaks shown in Fig. 5C against all protein profiles within the respective mass range(s) identified the cytochrome c oxidase subunit 7A-related protein (COX7R) as a constitutive OXPHOS subunit. Consistent with its previously described function as an assembly factor of OXPHOS super-complexes (27, 28), this 6.5-kDa oligopeptide was found specifically and stably associated with all OXPHOS super-complexes containing both complex III dimers and complex IV (Fig. 5C).

Finally, we extended the correlation analysis to the large set of remaining protein profile peaks identified by our csBN-MS approach to screen for novel protein assemblies. Among the many clusters identified (complete map in supplemental figure 2 and example profiles provided in supplemental figure 3), we found significant profile correlation of the pore-forming subunits of the anion channels from the outer mitochondrial membrane (VDAC isoforms 1–3) and the constituents of the translocase of the mitochondrial outer membrane (TOM complex), most prominently TOM20 (Fig. 6A). As these proteins are highly conserved among species, we set out to test whether an interaction between TOM subunits and the major VDAC homolog, Porin1, exists in yeast mitochondria. As shown by Western blot analysis in Fig. 6B, the previously described interaction partner OM14 (29) and the endogenous TOM subunits TOM20, TOM22, and TOM40 were specifically co-purified along with HA-tagged Porin1 (the major yeast VDAC homolog). Elution of the affinity-purified proteins under native conditions by an HA peptide and subsequent analysis by BN-PAGE revealed association of TOM40 and TOM22 in a 440-kDa complex with Porin1 (Fig. 6C, upper panel), consistent with the protein profile cluster in Fig. 6A. This finding was corroborated by specific “reverse” purification of Porin1-containing complexes via His-tagged TOM22 (Fig. 6C, lower left panel). To exclude that this 440-kDa complex represents just an assembly intermediate of Porin1 at the TOM complex, 35S-labeled Oxa1 was arrested at the TOM complex by dissociation of the membrane potential (24) followed by affinity purification of the HA-tagged Porin1. After separation of the purified proteins by BN-PAGE, autoradiography revealed the binding of Porin1 to translocation-competent TOM complexes (Fig. 6C, lower right panel).

Taken together, these results demonstrate the potential of the csBN-MS approach to resolve novel protein complexes and to identify their subunit composition with high precision and at proteomic scale.

**DISCUSSION**

In this study we present a high resolution BN-MS workflow developed for characterization of native protein complexes at proteomic scale. Compared with other BN-MS approaches applied to comparable samples in previous studies (13, 14), csBN-MS offers several major advantages as follows: (i) it uses nearly the full resolving power of the BN-PAGE separation; (ii) it provides detailed abundance-mass profiles over an extended molecular size range, and thus (iii) it resolves the
Fig. 5. Analysis of respiratory chain complexes by csBN-MS. A, abundance\textsubscript{norm} mass profiles (arbitrary units) determined for the canonical subunits of the ATP synthase (OXPHOS complex V). Note the identification of at least five different complex assemblies (indicated by arrows) over the investigated mass range (obtained from the mass calibration in Fig. 4C). Color-coding of the protein subunits is indicated on the right. Inset, boxed section at enlarged scale; note that all known subunits were identified and quantified despite the low abundance of this high molecular weight assembly. The abundance\textsubscript{norm} values of the profiles suggest that all assemblies display the same subunit composition except for the complex at $\sim$280 kDa, which represents the F1 catalytic core only. B, abundance\textsubscript{norm} mass profiles of the subunits of cytochrome c oxidase (complex IV) covering its monomeric and dimeric forms. The core subunits COX1 and COX2 (red) were found in at least four monomer sub-populations (arrows) associated with different pools of the other known subunits; molecular heterogeneity was also visible in the dimeric form of complex IV. C, relative abundance mass profiles of respiratory chain complexes I–V. Complexes (indicated in black) and super-complexes (indicated in purple) were assigned based on the high resolution subunit profiles and the work by (2). Unbiased correlation analysis of the super-complex assemblies with the profiles of all other mitochondrial proteins (see “Experimental Procedures”) identified COX7R (red) as a selective subunit of super-complexes containing both complexes III\textsubscript{2} and IV.
Fig. 6. Identification of super-complexes assembled from VDACs/Porins and TOM complexes. A, relative abundance-mass profiles of VDAC1–3 and TOM20 proteins. Color-coding and the Pearson correlation coefficient r of profile peaks are shown on the right. B, interaction of yeast Porin1 and the TOM complex detected by affinity purification. Mitochondria isolated from wild-type (WT) or yeast strain expressing HA-tagged VDAC homolog Porin1 (Por1HA) were subjected to affinity purification via anti-HA chromatography. Load (0.75%) and elution (100%) fractions were separated by SDS-PAGE, and proteins were detected by Western blotting with the indicated antisera. The Porin1 interactor OM14 and several TOM proteins (upper panel) were specifically co-purified, whereas three unrelated control proteins (lower panel) were not detected in the eluates. C, upper panel, affinity purifications as in B eluted under non-denaturing conditions and resolved by BN-PAGE. Western blot detection with anti-TOM40 (left) and anti-TOM22 (right) antibodies identified full-size TOM complex (around 450 kDa) specifically co-purified with HA-Porin1. Lower left panel, wild-type (WT) and Tom22His mitochondria subjected to nickel in complex with nitrilotriacetic acid purification followed by separation via BN-PAGE and Western blotting. Porin complexes were detected using anti-Porin1 antibodies (load 0.75%, elution 100%). Lower right panel, wild-type (WT) and Por1HA mitochondria were incubated with 35S-labeled Oxa1 in the absence of a membrane potential to arrest the precursor at the TOM complex. Subsequently, samples were subjected to anti-HA chromatography, and protein complexes were separated by BN-PAGE. 35S-Labeled Oxa1 arrested at the TOM complex was visualized with digital autoradiography. Note the increase in apparent mass of TOM complexes due to the stalled Oxa1 substrate.
Resolution of csBN-MS—The high resolution of the approach is achieved by the high "sampling rate" that is enabled by the cryo-micromtome-assisted slicing of BN gels and that can be adjusted to step sizes from 0.1 to 0.5 mm (Figs. 1 and 2). It must be pointed out that homogeneity of protein migration in the BN gel and distortion-free alignment of the gel lane by the stained protein bands during the embedding procedure (Fig. 2) are prerequisite for effective high resolution sampling. These factors become even more critical when processing broader gel lanes and might set a practical limit to the sampling step size. In this benchmark study, we chose 0.3 mm also to limit the number of subsequent analyses but to still cover the sharpest-focusing proteins and/or protein complexes by at least five LC-MS/MS measurements (Fig. 4). Indeed, this setting led to fully resolved profiles with a 2-3-fold "over"-sampling when related to the minimal observed peak FWHM of 1 mm (reflecting the resolution limit of the used BN gel, Fig. 1). The high size resolution is key for the success of BN-MS-based complexome profiling for several reasons. First, redundancy of MS data can be used to eliminate errors occurring during LC-MS/MS analysis or signal assignment, resulting in more reliable quantitative information, which otherwise would require replicate measurements. Second, it reduces the complexity in individual samples, thereby increasing the dynamic range and protein coverage as demonstrated for the OXPHOS complex subunits (Fig. 5). Third, it allows for identification of previously unrecognized molecular heterogeneities of complexes (e.g. cytochrome c oxidase) and separation of overlapping complex populations (Fig. 5). Finally, the higher size resolution strongly enhances the validity of protein profile correlation and/or clustering approaches for the identification of protein complexes.

Such global Pearson correlation with hierarchical clustering is commonly used to define protein complexes in BN-MS studies (13, 14), although this method is in fact rather unsuited for identification of complex protein interactions. It performs reasonably well for dominant and exclusive protein assemblies, but it usually fails for proteins that are part of different complexes or for complexes with heterogeneous subunit composition. The latter is immediately evident from the marked number of false-positive and false-negative protein constituents in correlation clusters assigned to the known OXPHOS complexes I–V in previous studies (detailed in the supplemental material of Refs. 13, 14), as well as from the example of COX7R that was identified and well resolved in these studies but escaped assignment to OXPHOS complexes due to its specific incorporation into distinct supercomplexes (Fig. 5) (see the supplemental material in Ref. 13). As an alternative approach to analyze protein mass-abundance profiles, we used automatized Gaussian fitting to identify protein populations ("complex peaks") that were subsequently screened for unbiased correlation with profiles of other proteins in the corresponding size range. This procedure identified unexpected association of voltage-dependent anion-selective channel proteins (VDAC) 1–3 with the TOM complex, which forms the general entry gate for most of the mitochondrial proteins (Fig. 6). Further examples of putative complexes and profile clusters are given in supplemental figure 3.

Comprehensiveness of csBN-MS—A decisive feature of csBN-MS besides resolution is the comprehensiveness provided by the approach for both the number of retrieved protein profiles as well as the number of identified complexes. Thus, around 70% of the known mitochondrial proteome (according to the MitoCarta resource and UniProtKB/Swiss-Prot database) were unambiguously identified and reliably quantified over the mass range from 100 to 3,500 kDa. Notably, the complete set of the 90 OXPHOS building blocks (except the NU6M subunit of complex I, which does not provide tryptic peptides accessible to the LC-MS setup used) was identified, including the "QCR11" subunit of complex III representing a short N-terminal cleavage product (amino acids 1–78) of the UCP/Rieske subunit precursor (30) and the dynamic ATP synthase interactor ATIF1 (26). These subunits could be assigned to at least 13 separate (super-)complexes and revealed both, modules with rather fixed subunit composition such as ATP synthase (complex V) and highly heterogeneous entities like cytochrome c oxidase (complex IV) (Fig. 5, A and B) in line with the plasticity model of the OXPHOS complexes (31). Thus, csBN-MS opens a way to study the stoichiometry of the OXPHOS (super-)complexes in a precise and quantitative manner to challenge the opposing models for structural organization and function of the dynamic entity (31, 32). The high resolution of the method is also of interest for resolving sub-complexes and assembly intermediates of the (super-)complexes. In addition, we identified COX7R as a specific interaction partner of complex III2–IV-containing super-complexes (Fig. 5). This 6.5-kDa oligopeptide has recently been identified as an essential assembly factor of super-complexes which, when abolished, causes respiratory deficits (27, 28). Interestingly, COX7R showed a fixed abundance relation to complex IV (in all OXPHOS super-complexes containing also a complex III dimer, Fig. 5C) suggesting that this protein is another stably associated subunit of certain cytochrome c oxidase super-complexes rather than a classical assembly factor.

* This work was supported by Deutsche Forschungsgemeinschaft Grants BE4679/2-1 and SFB 746/TP22 (to T.B.), SFB 746/TP16 and SFB TRR 152/TP02 (to B.F.), and SFB 746/TP20 and SFB TRR 152/TP05 (to U.S.). The authors declare that they have no conflicts of interest with the contents of this article.

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REFERENCES

1. Schägger, H., and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. 195, 223–231

2. Schägger, H., and Pfeiffer, K. (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J. 19, 1777–1783

3. Wittig, I., and Schägger, H. (2009) Native electrophoretic techniques to identify protein–protein interactions. Proteomics 9, 5214–5223

4. Reisinger, V., and Eichacker, L. A. (2007) How to analyze protein complexes by 2D blue native SDS-PAGE. Proteomics 7, 6–16

5. Devreese, B., Vanrobaeys, F., Smet, J., Van Beeumen, J., and Van Coster, R. (2002) Mass spectrometric identification of mitochondrial oxidative phosphorylation subunits separated by two-dimensional blue-native polyacrylamide gel electrophoresis. Electrophoresis 23, 2525–2533

6. Meyer, B., Wittig, I., Trifilieff, E., Karas, M., and Schägger, H. (2007) Identification of two proteins associated with mammalian ATP synthase. Mol. Cell. Proteomics 6, 1690–1699

7. Klodmann, J., Senkler, M., Rode, C., and Braun, H. P. (2011) Defining the protein complex proteome of plant mitochondria. Plant Physiol. 157, 587–598

8. D’Amici, G. M., Rinalducci, S., Murgiano, L., Italiano, F., and Zolla, L. (2010) Oligomeric characterization of the photosynthetic apparatus of Rhodobacter sphaeroides R26.1 by non-denaturing electrophoresis methods. J. Proteome Res. 9, 192–203

9. Selao, T. T., Branca, R., Chae, P. S., Lehtio, J., Gellman, S. H., Rasmussen, S. G., Nordlund, S., and Nören, A. (2011) Identification of chromatophore membrane protein complexes formed under different nitrogen availability conditions in Rhodospirillum rubrum. J. Proteome Res. 10, 2703–2714

10. Behrens, C., Blume, C., Senkler, M., Eubel, H., Peterhansel, C., and Braun, H. P. (2013) The ‘protein complex proteome’ of chloroplasts in Arabidopsis thaliana. J. Proteomics 91, 73–83

11. Li, Z., Xu, L., Li, F., Zhou, Q., and Yang, F. (2011) Analysis of white spot syndrome virus envelope protein complexome by two-dimensional blue native native/SDS PAGE combined with mass spectrometry. Arch. Virol. 156, 1125–1135

12. Wessels, H. J., Vogel, R. O., van den Heuvel, L., Smetink, J. A., Rodenburg, R. J., Nijtmans, L. G., and Farhoud, M. H. (2009) LC-MS/MS as an alternative for SDS-PAGE in blue native analysis of protein complexes. Proteomics 9, 4221–4228

13. Heide, H., Bleier, L., Steger, M., Ackermann, J., Dröse, S., Schwamb, B., Zörnig, M., Reichert, A. S., Koch, I., Wittig, I., and Brandt, U. (2012) Complexome profiling identifies TMEM126B as a component of the mitochondrial complex I assembly complex. Cell Metab. 16, 536–549

14. Wessels, H. J., Vogel, R. O., Lightowers, R. N., Spellbrink, J. N., Rodenberg, R. J., van den Heuvel, L. P., van Gool, A. J., Groech, J., Smetink, J. A., and Nijtmans, L. G. (2013) Analysis of 953 human proteins from a mitochondrial HEK293 fraction by complexome profiling. PLoS One 8, e68340

15. Schwenk, J., Harmel, N., Brechet, A., Zolles, G., Berkerfeld, H., Müller, C. S., Bildi, W., Baehrens, D., Huber, B., Kluk, A., Klöcker, N., Schulte, U., and Fakler, B. (2012) High resolution proteomics unravel architecture and molecular diversity of native AMPA receptor complexes. Neuron 74, 621–633

16. Turecek, R., Schwenk, J., Fritzius, T., Ivankova, K., Zolles, G., Adelfinger, L., Jacquier, V., Besseyrias, V., Gassmann, M., Schulte, U., Fakler, B., and Bettler, B. (2014) Auxiliary GABA_A receptor subunits uncouple G protein ρ1 subunits from effector channels to induce desensitization. Neuron 82, 1032–1044

17. Rendon, A., and Masmoudi, A. (1985) Purification of non-synaptic and synaptic mitochondria and plasma membranes from rat brain by a rapid Percoll gradient procedure. J. Neurosci. Methods 14, 41–51

18. Pandey, A., Andersen, J. S., and Mann, M. (2000) Use of mass spectrometry to study signaling pathways. Sci. STKE 2000, pl1

19. Bildi, W., Haupt, A., Muller, C. S., Biniossek, M. L., Thumfart, J. O., Huber, B., Fakler, B., and Schulte, U. (2012) Extending the dynamic range of label-free mass spectrometric quantification of affinity purifications. Mol. Cell Proteomics 11, M111.07955

20. Cox, J., Michalski, A., and Mann, M. (2011) Software lock mass by two-dimensional minimization of peptide mass errors. J. Am. Soc. Mass Spectrom. 22, 1373–1380

21. Meisinger, C., Ryan, M. T., Hill, K., Model, K., Lim, J. H., Sickmann, A., Müller, H., Meyer, H. E., Wagner, R., and Pfanner, N. (2001) Protein import channel of the outer mitochondrial membrane: a highly stable Tom40-Tom22 core structure differentially interacts with preproteins, small tom proteins, and import receptors. Mol. Cell. Biol. 21, 2337–2348

22. Wenz, L. S., Opalinski, L., Schulter, M. H., Ellenrieder, L., Ieva, R., Böttger, L., Qiu, J., van der Laan, M., Wiedermann, N., Guiard, B., Pfanner, N., and Becker, T. (2014) The presequence pathway is involved in protein sorting to the mitochondrial outer membrane. EMBO Rep. 15, 678–685

23. Meisinger, C., Pfanner, N., and Truscott, K. N. (2006) Isolation of yeast mitochondria. Methods Mol. Biol. 313, 33–39

24. Frazier, A. E., Chacinska, A., Truscott, K. N., Guiard, B., Pfanner, N., and Rehling, P. (2003) Mitochondria use different mechanisms for transport of multispanning membrane proteins through the intermembrane space. Mol. Cell. Biol. 23, 7818–7828

25. Wittig, I., Beckhaus, T., Wumaier, Z., Karas, M., and Schägger, H. (2010) Mass estimation of native proteins by blue native electrophoresis: principles and practical hints. Mol. Cell Proteomics 9, 2149–2161

26. Schwerzmann, K., and Pedersen, P. L. (1986) Regulation of the mitochondrial ATP synthase/ATPase complex. Arch. Biochem. Biophys. 250, 1–18

27. Ikeda, K., Shiba, S., Horie-Inoue, K., Shimokata, K., and Inoue, S. (2013) A stabilizing factor for mitochondrial respiratory supercomplex assembly regulates energy metabolism in muscle. Nat. Commun. 4, 2147

28. Lapuente-Brun, E., Moreno-Loshuertos, R., Acín-Pérez, R., Latorre-Pellícer, A., Colás, C., Balsa, E., Perales-Clemente, E., Quirós, P. M., Calvo, E., Rodríguez-Hernández, M. A., Navas, P., Cruz, R., Carracedo, A., López-Otin, C., Pérez-Martos, A., Fernández-Silva, P., Fernández-Vizarra, E., and Enríquez-Jorquera, J. A. (2013) Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. Science 340, 1567–1570

29. Lauffer, S., Mäbert, K., Czupalla, C., Porsche, T., Hoflack, B., Rödel, G., and Krause-Buchholz, U. (2012) Saccharomyces cerevisiae porin pore forms complexes with mitochondrial outer membrane proteins Om14p and Om45p. J. Biol. Chem. 287, 17447–17458

30. Brandt, U., Yu, L., Yu, C. A., and Trumpower, B. L. (1993) The mitochondrial targeting presequence of the Rieske iron–sulfur protein is processed in a single step after insertion into the cytochrome bc1 complex in mammals and retained as a subunit in the complex. J. Biol. Chem. 268, 8387–8390

31. Acín-Pérez, R., Fernández-Silva, P., Peleato, M. L., Pérez-Martos, A., and Enríquez-Jorquera, J. A. (2008) Respiratory active mitochondrial supercomplexes. Mol. Cell 32, 529–539

32. Genova, M. L., and Lenaz, G. (2014) Functional role of mitochondrial respiratory supercomplexes. Biochim. Biophys. Acts 1837, 427–443