Chapter 3

A search for peroxisomal membrane protein complexes by BN-PAGE, Mass Spectrometry and Electron Microscopy

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ABSTRACT

In the last decades many of the Pex proteins, involved in protein transport over the peroxisomal membrane, have been genetically and biochemically characterized in detail and models of their interactions have been proposed. However, the models are not based on direct structural studies, such as single particle electron microscopy, which means that there are still many unsolved problems related to these large complexes. We explored the use of Blue-Native Gel electrophoresis (BN-PAGE), which is one of the best methods to purify large, transient membrane complexes, and in combination with biochemical characterization, and mass spectrometry looked for ways to assign subunit compositions and interactions. Here, we have performed a pilot study of the major peroxisomal membrane complexes in the yeast species Saccharomyces cerevisiae and Hansenula polymorpha. We have monitored the presence and purification of large, hypothetical Pex complexes by using western blotting with specific antibodies against Pex14p. However, upon 1D and 2D/SDS Blue Native gels electrophoresis from peroxisomal fractions only few protein bands could be attributed to peroxisomes. In fact, no large Pex protein complexes were found, which we also proved by mass spectrometry. This could be related to a very low amount of peroxisomal membrane proteins compared to mitochondrial proteins. Peroxisomes were obtained by differential and sucrose density centrifugation and mitochondria were the major contaminating organelles in purified peak fractions. It appears that further purification of the initial membranes is crucial. This requires more advanced techniques and better separation protocols to separate peroxisomes from mitochondria.
INTRODUCTION

Eukaryotic cells are characterized by the presence of organelles that are involved in various metabolic processes (e.g. mitochondria, Golgi apparatus etc.). Microbodies including peroxisomes also belong to this group. They are ubiquitous organelles involved in numerous catabolic and anabolic pathways in eukaryotic cells (Van den Bosch et al. 1992). Their biogenesis can be conceptionally divided into different functional aspects including organelle segregation during the course of cell division, membrane biogenesis and import of matrix proteins (Kunau 1998). The function of peroxisomes depends on the organism, tissue, developmental stage and environmental conditions, like in the case of yeast and fungi.

In general, there are only low numbers of enzymes associated with the microbody membrane. Sulter and co-workers (1993) showed by freeze fracture electron microscopy that the peroxisome membranes possess a very low content of large integral membrane proteins. Based on their function, these membranes must contain proteins involved in matrix protein import, membrane protein insertion, organellar maintenance and solute transport.

The major group of known peroxisome-bound proteins are peroxins. To date 32 different peroxins have been indentified (Vizeacoumar et al. 2004; Van der Zand et al. 2006). Several of these proteins have been predicted to appear in larger homomeric or heteromeric complexes (Sacksteder and Gould 2000; Subramani et al. 2000; Agne et al. 2003). Many of the known peroxins are essential for peroxisomal matrix protein import. Both Pex5p and Pex7p are the import receptors that specifically recognize PTS1 and PTS2, respectively. PTS receptors are predominantly soluble proteins that bind their cargo proteins in the cytosol and then target them to the peroxisomal membrane, from where the matrix proteins are imported into the organelle and then the free receptors are recycled back to the cytoplasm (Marzioch et al. 1994; Dodt and Gould 1996). The steps in which the import receptor is membrane associated can be subdivided into three distinct stages: (I) docking of the receptor to the recognition machinery at the membrane (consisting of at least Pex13p, Pex14p and Pex17p), (II) transfer and release of cargo proteins and (III) dissociation of the receptor from the organelle into the cytoplasm (by Pex4p and Pex22p).
It is well established that Pex13p, Pex14p, and Pex17p contribute to or mediate the docking of the receptors to the *cis*-side of the peroxisomal membrane (Subramani et al. 2000; Holroyd and Erdmann 2001). Pex13p and Pex14p recognize and physically bind both of the import receptors, Pex5p and Pex7p. Lutz (2004) showed by using BN-PAGE that Pex14p was present as a protein complex of approximately 450-550 kDa and 900 kDa in *H. polymorpha* and *S. cerevisiae*, respectively.

A second group of membrane proteins consist of Pex2p, Pex10p, and Pex12p. These three peroxins are integral membrane proteins of peroxisomes and contain a RING zinc finger at the C-terminal part. Impaired function of these three peroxins results in the failure of matrix protein import, implying that RING peroxins are involved in the process of protein transport across the membrane, possibly as members of import machinery constituents. Moreover, Pex2p, Pex10p, and Pex12p are mutually distinct in their primary structure except for a commonly shared RING finger (Okumoto et al. 2000). Thus, the RING finger is probably important for the function of these peroxins. The RING finger domains to the *cis* side of the peroxisomal membrane (Holroyd and Erdmann, 2001) exhibit pairwise interactions, and bind the PTS1 receptor Pex5p (Chang et al. 1999). Association of these two subcomplexes into a large complex was observed. Moreover, epistasis analysis predicts that during the peroxisomal import process the RING finger peroxins act downstream of Pex13p, Pex14p, and Pex17p (Collins et al., 2000). This hypothesis predicts that the three RING finger peroxins function together and are involved in the translocation step for receptors and cargo (Gould and Collins 2002). Five other membrane bound peroxins, Pex1p, Pex6p, Pex4p, Pex22p, and Pex10p are reported to act subsequently to the RING finger peroxins and thus are implicated in posttranslocation events (Collins et al. 2000).

Although biochemical studies of peroxisomal protein complexes suggest many interactions, there are still many open questions which need to be solved about the spatial organization of these subcomplexes, even on the low-resolution level. One of the ways to determine subunit interaction is to use single particle electron microscopy, which is a well-established technique to obtain structural information about large biomolecules at a resolution of 20 Å, or better. Schliebs et al. 1999 showed the structure Pex5p from human and several years later Moscicka et al. 2007 determined the structure of the Pex5p-Pex20p complex.
In this study we aim to analyse and identify protei n complexes at the peroxisomal membranes of the two yeast species *Saccharomyces cerevisiae* and WT *Hansenula polymorpha* using blue native gel electrophoresis (BN-PAGE), mass spectrometry and electron microscopy.

Although we were able to isolate the peroxisome and detect the presence of Pex14p by antibodies in isolated fractions, we did not detect any large Pex complex present after protein solubilization. One of the reasons why it is difficult to detect such complexes is the fact that peroxisomal membrane protein complexes are difficult to separate from the much more abundant mitochondrial membrane complexes. Because we think that it is worth to continue attempts to obtain pure fractions of the largest Pex complexes for structural analysis we present of our analysis in the context of such attempts.

**MATERIALS AND METHODS**

*Strains, media and growth conditions of S.cerevisiae and H.polymorpha*

The yeast strain used in this study was wild-type *ULT-7A* (Erdmann et al. 1991), producing ProtA-TEV-Pex14p. Complete and minimal media used for yeast culturing have been described previously (Erdmann et al. 1989). YNO medium contained 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract, and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0.

*Hansenula polymorpha* NCYC495 cells were grown at 37 °C on mineral medium (van Dijken et al. 1976) supplemented with 0.5 % (w/v) glucose or 0.5 % (v/v) methanol as a carbon source, and 0.25 % (w/v) ammonium sulfate as nitrogen source. Leucine was added at a final concentration of 30 µg ml⁻¹.

*Isolation of peroxisome protein complexes from S.cerevisiae by using IgG-Sepharose*

Oleate induced yeast cells were lysed according to Agne et al. 2003, using glass beads and lysis buffer (20 mM HEPES, 100 mM KOAc, 5 mM MgOAc [pH 7.5]) containing protease inhibitors (1mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM sodium azide, 2 µg/ml aprotinin, 0.35 µg/ml bestatin, 1 µg/ml pepstatin, 2.5 µg/ml leupeptin, 0.16 mg/ml
benzamidin, 5 µg/ml antipain, 5 mM sodium fluoride (NaF), 6 µg/ml chymostatin). Cell debris was sedimented, and the supernatant was centrifuged at 100,000 × g at 4 °C for 1 h. A protein concentration of 2.7 mg/ml was obtained. Membrane proteins were solubilized with the detergent digitonin (2% w/v final concentration) at 4°C for 1 hr. Unsolubilized material was removed by centrifugation (30 min., 18,000 × g). For affinity chromatography, the detergent extract was incubated with IgG-Sepharose (Pharmacia) for 12-14 h at 4°C overnight on a rotary shaker and the sedimentsed material was centrifuged at 5 min. at 4 °C (1500 x g). Bound material was collected, followed by 4x washing with 125 volumes of solubilization buffer containing 1/10 of the detergent concentration used for solubilization together with protease inhibitors. To elute bound protein complexes, 70 U TEV-protease (Invitrogen) and 21 µl solubilization buffer (containing 1/10 detergent concentration) were added to the Sepharose followed by incubation at 16 °C for 2 hr. TEV protease eluates were collected by centrifugation 1 min. (50 x g), and the elution was repeated twice. The native elution fractions were pooled and used directly or stored in small aliquots at −80 °C until use. The purity of subfractions was analyzed by SDS-PAGE followed by silver staining and subsequent Western blot analysis or by two-dimensional blue native PAGE.

**Isolation of peroxisomes from wt H. polymorpha by differential centrifugation and sucrose density centrifugation**

Methanol induced yeast cells were prepared for homogenization by using pre-incubation buffer (100 mM Tris-HCl [pH 8.0], 50 mM EDTA [pH 8.0] and 140 mM β-Mercaptoethanol), protoplast buffer (50 mM potassium phosphate [pH 7.2], 1.2 M sorbitol) and homogenization buffer (5 mM MES [pH 5.5], 0.1 mM EDTA, 1 mM KCl and 1 M sorbitol) respectively, containing protease inhibitors (1mM PMSF, 2 µg/ml aprotinin, 0.35 µg/ml bestatin, 1 µg/ml pepstatin, 2.5 µg/ml leupeptin, 5 µg/ml antipain). The resulting homogenate of protoplasts was used for differential centrifugation steps (as described by van der Klei 2000), and the organellar pellet was subjected to sucrose density centrifugation. The pellet was resuspended very carefully in a solution of 40% (w/w) sucrose in buffer B (containing 5 mM MES [pH 5.5], 0.1 mM EDTA, 1 mM KCl ) and the amount of protein loaded per gradient was 5 mg. Gradients were centrifuged for 2.5 hours and were harvested in 21 fractions. The organellar peak fractions were identified by performing alcohol oxidase (Verduyn et al. 1984) and cytochrome c oxidase (Douma et al. 1985) assays. The peroxisomal peak fractions were
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pooled together and the sucrose concentration was measured and lowered to 44%. The collected material was spun down for 30 min., 18,000 rpm. After spinning down, the supernatant was removed and the pellet was resuspended in lysis buffer [pH 7.5] (20 mM Hepes, 100 mM KOAc, 5 mM MgOAc) containing protease inhibitors (Complete™, 1mM PMSF, 2 µg/ml aprotinin, 0.35 µg/ml bestatin, 1 µg/ml pepstatin, 2.5 µg/ml leupeptin). To separate peroxisomal membranes from the peroxisomal matrix proteins, the pellet was centrifuged at 100,000 × g at 4 °C for 1 h. The pellet fraction containing peroxisomal membranes were stored in small aliquots at -80°C until use.

*Gel electrophoresis procedures and immunoblotting*

Protein concentrations were determined using the Bio-Rad Protein Assay system (Biorad GmbH, Munich, Germany) with bovine serum albumine as a standard. Proteins were separated by standard SDS-PAGE or one-dimensional Blue Native PAGE according to standard procedures (Schägger and von Jagow 1987). For subunit analysis, gel stripes including the resolved protein complexes were transferred horizontally onto a second gel dimension, which was carried out in the presence of SDS (two-dimensional Blue native/SDS-PAGE). Proteins were visualized by Coomassie Blue-colloidal staining (Neuhoff et al. 1985 and 1990), silver stain or blotted onto nitrocellulose filters. Blots were incubated with Pex14p antibody from *H. polymorpha*. Selected protein complexes were cut out from Coomassie Blue-stained two-dimensional blue-native gels and subunits were identified by mass spectrometry.

*Electron Microscopy*

Negatively stained specimens were prepared with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM10 FEG electron microscope operated at 100 kV. Images were recorded at 34000 x magnification with a pixel size (after binning the images) of 3.75 Å at the specimen level.
RESULTS

Purification and solubilization of Peroxin Complexes from S.cerevisiae

In order to investigate peroxisomal membrane protein complexes, we first performed an isolation procedure from total cellular membranes of *S.cerevisiae* without separating organelles. Peroxin-containing complexes were isolated by IgG affinity chromatography, utilizing TEV-ProtA-tagged peroxins. Previous work on protein complexes of the respiratory chain from mitochondrial membranes had demonstrated that the solubilization conditions have a significant impact on the composition and integrity of the isolated complexes (Schägger and Pfeiffer, 2000). We aimed here to test efficient solubilization conditions and as a criterion for solubilization, we employed a centrifugation step of 100,000 × g for 1 h. We chose to follow enrichment in Pex complexes by monitoring protein A fusion of Pex14p because that protein has been implicated in multiple interactions with other peroxins. ProtA-TEV-Pex14p was isolated via IgG-Sepharose chromatography from supernatants obtained after solubilization with 1% digitonin.

Data obtained by Agne and co-workers (2003) indicated that only solubilization of the membranes with digitonin gives sufficient amounts of apparently intact peroxisomal membrane protein complexes. Aliquots of TEV protease eluates, representing equal amounts of membranes, were separated by SDS-PAGE and stained with Coomassie or silver staining, and whole lanes were analyzed by mass spectrometry and subsequently compared with yeast database searches. Pex14p could be detected by Western blotting decorated by a specific antibody against Pex14p (Fig. 2A). However, mass spectrometry analysis on several gels from independent solubilization experiments did not indicate any (large) Pex complexes present in the eluates (Fig. 2B). However, an electron microscopy analysis indicated the presence of the dimeric ATP synthase from mitochondria (Fig. 1). This complex is easy recognizable because of its large size and specific shape (Dudkina 2006).

Analysis of fractions from *S. cerevisiae* from affinity purification by electron microscopy.

Negatively stained specimens of the solubilized peroxisomal fractions were found to contain a large contamination with dimeric ATP synthase molecules, which is a
mitochondrial supercomplex that can be recognized by its characteristic shape (Fig. 1). Further investigation by BN-PAGE gel electrophoresis and mass spectrometry proved that the fractions contained a lot of contamination with mitochondria proteins and no peroxisomal proteins were found.

**Figure 1.** Electron micrograph of a negatively stained membrane fraction after solubilization with 1% digitonin. The black boxes indicate ATP synthase dimers. Scale bar is 50 nm.

In a next set of experiments, affinity purification from a total cellular fraction was abandoned. Instead, organelles were first separated only by differential centrifugation, according to the methods section (purification and solubilization of Peroxin Complexes from *S. cerevisiae*). From the peroxisomal fraction, membrane solubilization was performed with 5% digitonin or with 10% n-dodecyl β-D-maltoside (DDM). The composition of generated fractions with 5% digitonin was tested by 1D blue native PAGE (Fig. 3). Several high molecular weight bands (MW) could be detected. The question now was to establish which of the protein bands observed after BN-PAGE are peroxisomal in nature. The reason for this is related to the fact that only a small portion of the total membrane proteins (approx. 5%, according to Janssen en Janssen 2001) represents peroxisomal proteins. This is in contrast to mitochondria, which contribute about 80% of the total membrane fraction. To check for the presence of specific mitochondrial complexes, solubilized membranes from mitochondrial peak fractions from *S. cerevisiae* were used as control (Fig. 3, lane 1). Comparison of the gel patterns indicates that some bands in the peroxisomal fraction run at a position where there is no strong profile of a mitochondrial protein, especially at the position of boxes 1, 5, 8 and 9 (Fig. 3, lane 2). Therefore 12 bands of interest were cut out and submitted to mass spectrometry (highlighted by boxes in Figure 3, lane 2). After detailed examinations, once again no Pex complexes were found in analysed bands. This suggest that an increase in
detergent concentration (from 1% to 5% digitonin) or the change to a stronger detergent (10% n-dodecyl β-D-maltoside, data not shown) could not induce the isolation of any large peroxisomal complex.

**Figure 2.** Pex14p-containing complexes from peroxisomal membranes analyzed by immunoblotting and mass spectrometry. Protein complexes were solubilized with 1% digitonin from isolated membranes of S. cerevisiae cells producing Pex14p-TEV-ProtA and purified via IgG-Sepharose affinity chromatography. (A) Western blot decorated using α-Pex14p antibody. The data show that a Pex14p signal was detected in peroxisomal membrane pellet (MP), E1 (elution) and E2. The presence of the Pex14p is indicated by the black boxes. In FW (flow thro) and W1-3 (washing) no signal is detected (as predicted). (B) A silver stained gel where the bands from elute fraction (lane E1 gel A) were cut and analyzed by mass spectrometry (pointed by arrows). Bands analyzed by mass spectrometry: K1 – ubiquinol-cytochrome-c reductase or H+ transporting two-sector ATPase (maybe Pex11p); K2 – TEV protease band; K3 – VDAC1, outer mitochondrial membrane protein porin 1; K4 – heat shock protein HSP60 precursor, mitochondrial; K5 – YSCE1B NIB; K6 – ubiquinol-cytochrome-c reductase; K7 – heat shock protein HSP60 precursor, mitochondrial; K8 – heat shock protein SSA1 or SSA2. In analyzed bands no Pex14p or other Pex proteins were detected.
Figure 3. Separation of peroxisomal protein complexes by BN-PAGE using a 4.5 - 16% gradient gel. The bands represent protein complexes found after solubilization of peroxisomal membranes from S. cerevisiae with 5% digitonin, in the supernatant after centrifugation to remove non-solubilized material. All visible bands were cut out and analysed by mass spectrometry. Boxes with numbers (1-12) mark bands analysed and assigned by mass spectrometry: 1-cytochrome c, 2-subunit 2 of ubiquinol cytochrome; 3-ATP I, beta subunit of ATPase; 4-HSP60; 5-Subunit of mitochondrial NAD(H) specific isocitrate dehydrogenase; 6-CDC19, Pyruvate kinase; 7-KGD1, component of the mitochondria alpha subunit; 8-Alcohol dehydrogenase isoenzyme; 9-CIT1, citrate synthase; 10-Acetyl CoA hydrolase; 11-Phosphoenolpyruvate carboxykinase; 12-RPS1, Ribosomal protein 10

Purification and solubilization of Peroxin Complexes from H. polymorpha

Since the investigations with S. cerevisiae gave only negative results, experiments were continued with starting material from H. polymorpha. In addition to the differential centrifugation step, an additional sucrose density gradient centrifugation was applied to improve the purification process. Organelles present in the homogenate of protoplasts prepared from methanol-grown H. polymorpha cells were separated by differential and sucrose density centrifugation (van der Klei 2000). Peroxisomal peak fractions were pooled and subjected to an osmotic shock, followed by centrifugation at 100,000 g in order to separate matrix components from the membranes. Several detergents were tested to solubilize peroxisomal membrane proteins. Aliquots of the washed membrane pellet were solubilized in
buffer containing digitonin or DDM (n-dodecyl β-D-maltoside) at different concentrations and were later centrifuged. A 1D Blue-native PAGE was carried out to monitor the protein complex composition from the peroxisomal yeast fractions of the sucrose gradient (data not shown), followed by 2D/SDS PAGE gel electrophoresis. The resulting bands were cut and analyzed by mass spectrometry. Peroxisomal fractions solubilized with 5% digitonin were submitted to 2D/SDS PAGE and visible spots (red circles) were analyzed by mass spectrometry (Figure 4B and C). Figure 4A presents a control experiment in which membranes isolated from mitochondrial peak fractions of *S. cerevisiae* were solubilized by 5% digitonin.

After detailed examinations by mass spectrometry, once again no large Pex complexes of interest, such as a docking complex composed of Pex14p, Pex17p, pex13p, were found in analysed spots. Nevertheless, the presence of several others peroxisomal proteins was detected (legend to figure 4). In parallel, we performed immunoblotting experiments with Pex14p (Fig. 2D) where we could detect its presence in the membrane fractions, but the observed molecular mass of 20 kDa suggests that the complex had already degraded during sample preparation. We have also used Pex10p antibody but the signal was not detected (data not shown). Our data clearly indicates that the membrane fractions were once again contaminated with mitochondrial proteins.
Figure 4. Purification of the peroxisomal complexes by sucrose gradient ultracentrifugation. Peroxisomal membrane proteins from yeast were solubilized by digitonin (5 g detergent per g protein) and resolved by ultracentrifugation as described under "Material and Methods". All fractions were analyzed by one-dimensional Blue native-PAGE (data not shown) to monitor their protein complex content. (A) Mitochondrial membrane protein complexes from S. cerevisiae solubilized by 5% digitonin. (B,C) Peroxisomal protein complexes were identified by parallel two-dimensional Blue native-SDS-PAGE and identified by mass spectrometry. Fractions 1 to 28ab contain respectively: 1- similarity to ubiquinol-cytochrome-c reductase 44K core protein; 2- ubiquinol-cytochrome-c reductase 40K chain II precursor; 3- F1F0-ATPase complex, F1 alpha subunit ; strong similarity to alpha subunit of H+transporting two-sector ATPase MGI2 - Kluyveromyces lactis; 4- mitochondrial F1F0-ATPase subunit beta ; strong similarity to F(1)F(0)-ATPase complex beta subunit ATP; 5- strong similarity to
transketolase – S. cerevisiae; 6- strong similarity to transketolase – S. cerevisiae; 7- transport protein USO1 – S. cerevisiae; 8- Porin/voltage-dependent anion-selective channel protein; strong similarity to mitochondrial outer membrane porin POR1/YNL055C – S. cerevisiae; 9- strong similarity to transketolase – S. cerevisiae; 10- strong similarity to TFIIIB 90 kDa subunit – S. cerevisiae; 11- identity to thymocyte nuclear protein 1; strong similarity to hypothetical protein – Mus musculus; 12- strong similarity to iso-1-cytochrome c – S. cerevisiae; 13- similarity to protein required for mitochondrial iron-sulfur cluster biosynthesis; strong similarity to hypothetical protein – S. cerevisiae; 14- Myosin-1 (Type II myosin); similarity to myosin-1 isoform (type II myosin) heavy chain S. cerevisiae; 15- strong similarity to SMC chromosomal ATPase family member – S. cerevisiae; 16a- similarity to integrin analogue gene USO1- S. cerevisiae; 16b- similarity to SYnthetic lethal with cdcForty – S. cerevisiae; Pre-mRNA-splicing factor syf2; 17- similarity to integrin analogue gene USO1- S. cerevisiae; 18- Myosin-1 (Type II myosin); similarity to myosin-1 isoform (type II myosin) heavy chain S. cerevisiae; 19- strong similarity to component of exocyst complex – S. cerevisiae; 20- strong similarity to a protein essential for assembly of a functional mitochondrial ATPase complex – S. cerevisiae; 21- no homology; weak similarity to AMP deaminase – S. cerevisiae; 22- Putative peroxiredoxin-A (Thioredoxin reductase) (Peroxisomal membrane protein A) (PMP20); strong similarity to peroxisomal membrane protein 20 CbPMP20 - Candida boidinii; 23- histone H3; strong similarity to histone H3 - Hypocrea jecorina; 24- Emp24p; strong similarity to component of the COPII-coated vesicles, 24 kDa EMP24/YGL200C – S. cerevisiae; 25- Putative peroxiredoxin-A (Thioredoxin reductase) (Peroxisomal membrane protein A) (PMP20); strong similarity to peroxisomal membrane protein 20 CbPMP20 - Candida boidinii; 26- strong similarity to nucleoside diphosphate kinase – S. cerevisiae; 27- Putative peroxiredoxin-A (Thioredoxin reductase) (Peroxisomal membrane protein A) (PMP20); strong similarity to peroxisomal membrane protein 20 CbPMP20 - Candida boidinii; 28- Myosin-1 (Type II myosin); similarity to myosin-1 isoform (type II myosin) heavy chain – S. cerevisiae. (D) Immunological localization of Pex14p detected by antibody against that protein.
DISCUSSION

In this report, we aimed to purify and characterize membrane-bound subcomplexes of the peroxisomal import machinery. These are the docking complex containing Pex14p, Pex17p, and Pex13p, and the RING finger complex comprising Pex2p, Pex10p, and Pex12p. We have tried to isolate the Pex14p complex from two different yeast species: *S. cerevisiae* and *H. polymorpha*. First we used the Pex14p-TEV-ProtA producing *S. cerevisiae* strain to isolate peroxin-containing complexes from peroxisomal membranes by IgG affinity chromatography. We chose the proteinA fusions of Pex14p as affinity target, because Pex14p has been implicated in multiple interactions with other peroxins. Secondly, with the same purpose of isolating peroxisomal membrane proteins, we used WT cells from *H. polymorpha*. In both methods, we have used BN-PAGE electrophoresis together with mass spectrometry and electron microscopy for the analysis and presence of membrane proteins in protein complexes. Our analysis shows the presence of Pex14p by Western Blots (Fig. 2A). Upon comparison the *S. cerevisiae* protein patterns in Coomasie stained BN-PAGE gels (Fig. 3) prepared from membranes of purified mitochondrial fractions (lane 1, Fig. 3) and total peroxisomal membrane (lane 2, Fig. 3) we detected a number of ‘peroxisomal’ protein bands which were different from mitochondrial bands. However, detailed analysis by mass spectrometry showed no presence of any Pex protein in the gel. The same results were obtained in a second experiment where we used WT cells from *H. polymorpha*. In 2D/SDS PAGE gels (Fig. 4B and C) from peroxisomal fractions solubilized by 5% digitonin and analysed by mass spectrometry we did not detect any major peroxisomal protein complexes, however the other peroxisomal proteins were detected. The Western Blot on 2D/SDS BN-PAGE gel (Fig. 4D) detected the presence of Pex14p but because the molecular weight of Pex14p is around 47 kDa the band which was observed, running at 20 kDa, probably contains a degradation product of Pex14.

Our analyses indicate that isolation of peroxin complexes from peroxisomes is not an easy task. It can be due to the well-known fact that peroxisomal membranes contain only low numbers of integral membrane components, in contrast to mitochondrial membranes, which are densely packed with large membrane proteins, with over 30 subunits for the yeast complex I. Hence it now appears crucial to purify such membranes to homogeneity, if possible, because otherwise they give a huge contamination. With classical methods like differential and sucrose centrifugation steps it is quite difficult to perform this, given the fact that
mitochondrial membrane proteins are possibly in the order of 50 times more abundant. Some novel cell and organelle separation methods will be discussed at the end of this discussion.

It is possible that our Pex protein complexes, followed by the marker Pex14p, were lost during sample preparation which includes 1) separation of peroxisomes by ultracentrifugation, 2) detergent solubilization, 3) removal of unsolubilized material by ultracentrifugation and 4) gel chromatography. Firstly, it is unlikely that whole peroxisomes are lost during ultracentrifugation, because the applied sedimentation force is strong enough to spin them down. Moreover, the presence of Pex14p was detected by immunoblotting before subjecting it to BN-PAGE electrophoresis (data not shown). Secondly, the most important step during sample preparation for BN-PAGE is adding the detergent solubilization. However, we cannot compare the samples before and after detergent solubilization (digitonin or DDM), because there are intact mitochondria in the fractions and they would close the slots of the BN gel so that nothing could enter into the matrix of the gel. We also checked different detergents under variable conditions and none of them worked fine. One further check could be to test the presence of the Pex14p by SDS-PAGE and Western blotting. Thirdly, we cannot make a full comparison after adding the detergent, because proteins remaining in the very last pellet after centrifugation could not be resuspended at all. Again, these proteins, in the form of aggregates, can not be separated on a BN gel, like mitochondria.

We conclude that the problem of the absence of Pex proteins is due to sample preparation before BN-PAGE, but that this conclusion was not so evident from previous work. For example, the method which was used by Marco Lutz (2004; in Chapter 3 of his Ph.D thesis) and Agne et al. (2003) did not differ substantially from ours, because the same detergents (digitonin and DDM) were applied and also similar buffers to isolate the peroxisomal membranes. However, Lutz did not show any Coomassie-stained 2D BN/SDS gels. Pex proteins only were detected by immunostaining of 2D BN/SDS gels, which does not tell us much about purity. Nevertheless, he detected several mitochondrial protein bands and he also showed that solubilization did not result in loss in Pex14p. However, we still cannot explain why we could not detect any Pex protein complexes after solubilization. Even, if we would specifically loose peroxisomal membranes during sample preparation, this still would not explain why we see so many mitochondrial proteins.
In general, the peroxisomal isolation and membrane solubilization need to be improved. The yield of the peroxisomes is still very low and the minor contamination with mitochondria in the peroxisomal peak fractions, leads to an overrepresentation of mitochondrial proteins due to the very high protein content of mitochondrial membranes which are too difficult to remove during the isolation procedures. There are several ways to apply separation methods which are not based on differential centrifugation. Hansel et al. (1991) proposed an Immunomagnetic Cell Separation system to separate neutrophils from eosinophils. Superparamagnetic particles were coupled to a monoclonal antibody against CD16, a molecule present on neutrophils but not on eosinophils. A peripheral blood granulocyte preparation, containing neutrophils and eosinophils, was incubated with these anti-CD16 particles. In the magnetic field of a permanent magnet, magnetically labelled neutrophils were then retained on columns with a ferromagnetic matrix. By this negative selection procedure, eosinophils of 99.5% purity were obtained. This method has been widely applied in biochemistry. Rodriguez-Paris et al. (1993) 5. J.M. Rodriguez-Paris, K.V. Nolta and T.L. Steck. *J. Biol. Chem.* (1993), pp. 9110–9116. View Record in Scopus Cited By in Scopus have used magnetic chromatography to separate lysosomes that were loaded with iron dextran by endocytosis. Another novel method is free-flow electrophoresis (FFE) which is used together with differential centrifugation and density gradient purification methods. FFE was used to increase the purity of mitochondrial isolates from *Arabidopsis thaliana* by separating them from plastids and peroxisomes according to differences in the surface charge of each organelle, yielding mitochondria that have seven times less contamination (Eubel et al. 2007). At a charge difference of 800 V, mitochondria, peroxisomes, and other cellular material could be separated in FFE, without overlap (Eubel et al. 2008). We expect that FFE will also work for separating yeast peroxisomes and mitochondria, if the optimal charge difference is found experimentally.

In conclusion, with novel separation methods the isolation procedure of peroxisomes could be improved in the near future. Together with novel purification protocols this will open new territories in the study of the structure of large, intact peroxisomal membrane complexes.

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