Sorting of matrix proteins to peroxisomes in the methylotrophic yeast Hansenula polymorpha
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Chapter 5

Protein complexes in the peroxisomal membrane visualized by blue native gel electrophoresis

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Abstract

Protein complexes in biological membranes function in a variety of processes, including protein translocation. For peroxisomes the presence of such protein complexes has been predicted from two-hybrid and immunoprecipitation experiments. Using blue native gel electrophoresis, we present evidence that several protein complexes of different sizes exist in the peroxisomal membrane of the yeast Hansenula polymorpha. Furthermore, the size and number of these complexes varied with the choice and concentration of detergent. One of the membrane proteins involved in peroxisomal matrix protein import, Pex14p, was demonstrated to be a component of a protein complex of approximately 400-500 kDa, together with a number of other proteins.

Introduction

Eukaryotic cells contain organelles (e.g. the nucleus, endoplasmic reticulum, Golgi apparatus and mitochondria) that are involved in various cellular processes. In the membranes of these organelles protein complexes are present, which are involved in several aspects of organelar maintenance and function. The last class of cell compartments that was discovered (in 1954) compromises the microbodies (peroxisomes, glyoxysomes, glycosomes). These organelles consist of a single membrane that surrounds a proteinaceous matrix. Despite their simple morphology, microbodies can perform various functions, ranging from crucial roles in the β-oxidation of very long chain fatty acids and biosynthesis of cholesterol in man, mobilization of storage oil and photorespiration in plant, glycolysis in trypanosomes, penicillin production in filamentous fungi, to involvement in the primary metabolism of various carbon and/or nitrogen sources in yeast (reviewed by [1] and [2]). The yeast peroxisomal membrane contains machineries, which are involved in matrix protein import, membrane protein insertion, organelar maintenance and solute transport. However, in contrast to other organelles, the presence of protein complexes in the peroxisomal membrane that mediate these processes has not been demonstrated, yet.

To date, 23 different genes involved in peroxisome biogenesis (PEX genes) have been cloned by functional complementation of peroxisome-deficient (pex) mutants (reviewed in [12, 13]). Molecular details on the function of the various peroxins in peroxisome biogenesis begin to emerge. Several of the yet known peroxins are essential for peroxisomal matrix protein import. This process requires the function of cytosolic receptors (Pex5p and Pex7p), a putative receptor/cargo docking site on the peroxisomal membrane (consisting of at least Pex13p, Pex14p and Pex17p), a putative translocation site (that may involve Pex10p and Pex12p functions [49]) and the receptor recycling mechanism (mediated by Pex4p and Pex22p [35, 56]). Since genetic analysis (two-hybrid system) and immunoprecipitation experiments suggested that several of the above peroxins physically
interact, it is thought that they might function in larger protein complexes. About the other processes involved in peroxisome biogenesis much less is known. At least four peroxins, Pex3p, Pex16p, Pex17p and Pex19p are thought to play a role in either the formation of peroxisomal membranes or in insertion of membrane proteins. However, the dynamics of these mechanisms and/or the function of the peroxins therein remain unknown. Recently, it has been demonstrated that the peroxisomal membrane protein Pex11p could form homodimers. In small, newly formed peroxisomes monomeric Pex11p is present, whereas Pex11p is predominantly dimeric in mature organelles. This finding implicates that the oligomeric state of Pex11p could play a role in peroxisome maturation [8]. Oligomerization of peroxisomal membrane proteins has also been observed for mammalian ABC transporters. The two-hybrid system and co-immunoprecipitation provided evidence that these proteins could form homo- and heterodimers [178].

In this study we aimed to analyze possible protein complexes in the peroxisomal membrane of the methylotrophic yeast Hansenula polymorpha using blue native gel electrophoresis (BN-PAGE). The potential of BN-PAGE was previously demonstrated by the separation and characterization of the protein translocases in the mitochondrial outer- and innermembrane [179-183] the endoplasmic reticulum [184] and chloroplasts [185]. We demonstrated that peroxisomal membranes of H. polymorpha contain several protein complexes. The results of these experiments are included in this paper.

**Material and methods**

**Strains and growth conditions**

*Hansenula polymorpha* wild type strainNCYC 495 *leu1.1* [156] was grown at 37 °C in mineral medium [135] supplemented with 0.5 % (w/v) glucose or 0.5 % (v/v) methanol as carbon source, and 0.25 % (w/v) ammonium sulphate as nitrogen source. Leucine was added to a final concentration of 30 μg.ml⁻¹.

**Solubilization of peroxisomal membrane proteins**

Peroxisomes were isolated from methanol-grown *H. polymorpha* cells by subjecting an organellar pellet to sucrose density centrifugation [54]. The organellar peak fractions were located by performing alcohol oxidase [136] (peroxisomal) and cytochrome c oxidase [160] (mitochondrial) assays. Protein concentrations were determined using the Biorad Protein Assay system (Biorad Gmbh, Munich, Germany) using BSA as a standard. The peroxisomal peak fractions from the gradient were pooled and subsequently diluted with an equal volume of 0.1 M Tris.HCl buffer pH 8.0, containing 1 mM PMSF, Complete™ (Boehringer Mannheim Gmbh, Germany) and 0.1 mM EDTA. The peroxisomal membranes were separated from the peroxisomal matrix proteins by centrifugation (15 min, 200,000 x g, 4 °C). The pellet, containing the membranes, was resuspended in 50 mM Tris.HCl buffer pH 8.0, containing 1 mM PMSF, Complete™ and 0.1 mM EDTA. To solubilize the proteins, the peroxisomal membrane suspension was diluted with an equal volume of solubilization buffer (50 mM Tris.HCl pH 8.0, 20 % (v/v) glycerol, 100 mM NaCl, 5 mM PMSF, Complete™, 0.1 mM EDTA) containing 2 times concentrated detergent, n-Dodecyl β-D-Maltoside (DDM), digitonin or Triton X-100. Membrane remnants and debris were removed by centrifugation (10 min, 14,000 x g, 4 °C).
Protein complexes in the peroxisomal membrane

**Fig. 1.** Sucrose gradient, prepared from a 30,000 x g, organellar pellet obtained from homogenized, methanol-grown *H. polymorpha* wild type cells, showing the distribution of the activities of the peroxisomal enzymes AO (▼) and the mitochondrial enzyme cytochrome c oxidase (○). AO and CAT activities are mainly present around a density of approximately 53 % (w/w) sucrose (fraction 7 – 11), indicating that these fractions contain the peroxisomes. Cytochrome c oxidase activity coincided with the protein peak at a density of around 43 % (w/w) sucrose (fraction 32 – 35), demonstrating that the mitochondria sedimented in these fractions. Sucrose concentrations (+) are expressed as percentages (w/w), the protein concentrations (o) as mg/ml. The specific activities of AO, CAT and cytochrome c oxidase are expressed as percentages of the activity in the peak fractions, which were arbitrarily set at 100.

*Blue native gel electrophoresis*

Blue native gel electrophoresis (BN-PAGE) was performed essentially as described previously [179, 186, 187]. 1/10 volume of 10 times concentrated Loading dye solution (0.1 M Bis Tris pH 7.0, 0.5 M ε-amino n-caproic acid, 5 % (w/v) Coomassie Brilliant Blue G250) was added to the solubilized peroxisomal membrane proteins and directly applied on a 6 to 13 % polyacrylamide gradient gel (approximately 100-150 μg protein per lane). Electrophoresis was performed at 100 V through the stacking gel and at 500 V through the separation gel, at a temperature of 4 °C. After electrophoresis, the gel was subjected to either silver staining, Western blotting [137] or second dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [138]. Prior to Western blotting the blue native gel was soaked in SDS-PAGE electrophoresis buffer [138] for 10 min, before transfer to PVDF membrane (Millipore) [137]. For two-dimensional gel analysis, slices were cut from the first dimension blue native gel and layered on top of a second dimension SDS-PAA gel. After electrophoresis, gels were silver stained, or the proteins were blotted onto nitrocellulose membranes. Proteins on the nitrocellulose or PVDF blots were decorated using specific polyclonal antibodies against various *H. polymorpha* proteins and detected using the chemiluminescent Western blot kit (Boehringer Mannheim GmbH, Germany).
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Fig. 2. Solubilization of Pex3p and Pex14p from peroxisomal peroxisomal membranes. (A) Purified peroxisomes (P), the soluble fraction (S) and the membrane pellet fraction (M) were subjected to Western blotting using specific antibodies against Pex3p, Pex14p, AO and DHAS. These blots demonstrate that bulk of the peroxisomal membrane proteins Pex3p and Pex14p was detected in the membrane pellet fraction, while majority of the matrix proteins AO and DHAS was detected in the soluble fraction. (B) The peroxisomal membrane pellet fraction was first resuspended with 50 mM Tris.HCl buffer pH 8.0 containing the individual components of the solubilization buffer, 10 % (v/v) glycerol or 50 mM NaCl. Westernblot analysis using specific antibodies against Pex3p and Pex14p showed that these proteins were still associated to the membranes in the pelletable fraction (p) after centrifugation for 15 min at 200.000 x g, 4 °C. (C) However, after resuspension of the peroxisomal membrane fraction in solubilization buffer containing a detergent (0.5 %, 1 % (w/v) DDM, 0.2 %, 1 % (v/v) Triton X-100, or 1 % (w/v) digitonin) Pex3p and Pex14p were detected in the solubilized fraction (s). Equal portions of the pellet (p) and soluble (s) fraction were loaded per lane.

Results

Solubilization of the peroxisomal membrane proteins Pex3p and Pex14p

Blue native gel electrophoresis (BN-PAGE), which makes use of Coomassie dye to introduce negative charges on proteins, was reported as a powerful application for the separation and size determination of membrane protein complexes, which could be further characterized by a second dimension denaturing SDS-PAGE. BN-PAGE was first used by Schägger et al. [186] to characterize respiratory chain protein complexes from purified mitochondria. We adapted this technique to analyze the presence of protein complexes in the peroxisomal membrane of H. polymorpha wild type cells.

To isolate peroxisomes, a homogenate of methanol-grown H. polymorpha wild type cells was subjected to differential centrifugation to obtain an organellar pellet (30 000 x g pellet). After sucrose density centrifugation of the 30 000 g pellet, peroxisomes sedimented at approximately 53 % (w/w) sucrose (Fig. 1). The peroxisomal peak fractions were pooled and subsequently diluted with an equal volume of 0.1 M Tris.HCl buffer pH 8.0 to disrupt the organelles. The membranes were separated from the soluble fraction by centrifugation at 200.000 x g for 15 min. Western blot analysis showed that after centrifugation bulk of the peroxisomal membrane protein, Pex3p, and the membrane associated protein, Pex14p, were detected in the pelleted membrane fraction whereas, as expected, the matrix proteins alcohol oxidase (AO) and dihydroxy-acetone synthase (DHAS) were predominately soluble (Fig. 2A). The minor amounts of AO and DHAS present in the pellet fraction most likely represent protein that has become associated with the membrane, a phenomenon that has been observed before.

The membrane pellet was first resuspended in solubilization buffer without detergent to analyze the influence of the individual components of the solubilization buffer, 10 % (v/v) glycerol and 50 mM NaCl, on the peroxisomal membrane proteins Pex3p and Pex14p. As is evident from Fig. 2B both membrane proteins remained pelletable under these conditions,
indicating that these proteins were tightly associated with the peroxisomal membranes. However, upon addition of a detergent (0.5 and 1 % (w/v) DDM; 0.2 and 1 % (v/v) Triton X-100; 1 % (w/v) digitonin), Pex3p and Pex14p were solely detected in the supernatant, implicating that they became solubilized under these conditions (Fig. 2B). At enhanced concentrations of Triton X-100 (1 % (v/v)), Pex3p was no longer detectable, which suggests that the protein was apparently unstable and degraded. At lower concentrations, 0.2 % (v/v) Triton X-100, the level of Pex3p had already decreased significantly. In contrast to Pex3p, Pex14p was unaffected by the treatment with 1 % (v/v) Triton X-100. This indicates that some peroxins or membrane proteins, as exemplified by Pex3p and Pex14p, require different solubilization conditions.

Complexes of different sizes are present in peroxisomal membranes

The proteins, solubilized from peroxisomal membranes with 1 % (w/v) DDM, 1 % (v/v) and 0.2 % (v/v) Triton X-100, and 1 % (w/v) digitonin, were loaded on a BN-PAGE gel with a 6 to 13 % polyacrylamide gradient. After electrophoresis and silver staining approximately 10 different protein bands (designated as I to X; Fig. 3) could be observed ranging from approximately 150 kDa to over 700 kDa.
Protein complexes in the peroxisomal membrane

Fig. 5. Protein composition of protein complex VIII, IX and X. To compare components of different complexes, the lanes of the second dimension SDS-PAGE gels of protein complex VIII, X and IX are shown next to each other. Complex VIII (600 kDa) contained the same components (a - h) after solubilization with 1 % (w/v) DDM and 0.2 % (v/v) Triton X-100. Complex X (>700 kDa) obtained after solubilization with 1 % (w/v) digitonin demonstrated a similar protein pattern as protein complex VIII. Complex IX was solubilized using 1 % (w/v) DDM, 0.2 % (v/v) Triton X-100 and 1 % (w/v) digitonin, and contained the components a - k. Overlapping proteins from other complexes are indicated by (*).

This suggests that several protein complexes are present in the peroxisomal membrane. Also, their size and number seems to vary with the choice and concentration of detergent (see also Table I). For example, after solubilization of the membrane proteins with 1 % (w/v) DDM, at least 6 protein bands could be observed (Fig. 3). These protein bands were designated as I, II, IV, VI, VII, and IX. Using 1 % (v/v) Triton X-100 as a detergent, only 4 protein bands, III, V, VII, and IX are detectable, while at a lower concentration of Triton X-100 (0.2 % (v/v)) an additional protein band, VIII was present. Apparently, this protein complex is unstable at higher Triton X-100 concentrations. This phenomenon could for instance be caused by the dissociation or instability of one or more of its components, as could be observed for

Fig. 4. Second dimension analysis of protein complexes using SDS-PAGE. Lanes of a BN-PAGE gel containing proteins solubilized in 1 % (w/v) DDM (A), 1 % (w/v) digitonin (B) and 0.2 % (v/v) Triton X-100 (C) were cut out and layered on top of a SDS-PAGE gel. After electrophoresis, the SDS-PAGE gels were subjected to silver staining. As a reference for the first dimension, slices of a BN-PAGE gel (Fig. 3) were included in this figure. The components of Complex VIII (A and C) are indicated with a - h, the components of Complex IX with a - k, and the components of Complex X with a - f.
<table>
<thead>
<tr>
<th>Protein band</th>
<th>Estimated Mw (kDa)</th>
<th>Detergent</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>150</td>
<td>1 % DDM</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>175</td>
<td>1 % DDM</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>200</td>
<td>0.2 % Triton</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>250</td>
<td>1 % DDM</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>300</td>
<td>0.2 % Triton</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>350</td>
<td>1 % DDM</td>
<td>-</td>
</tr>
<tr>
<td>VII</td>
<td>450</td>
<td>1 % DDM, 1 % digitonin, 0.2 % Triton</td>
<td>Homo-oligomer</td>
</tr>
<tr>
<td>VIII</td>
<td>650</td>
<td>1 % DDM, 0.2 % Triton</td>
<td>8 (a - h)</td>
</tr>
<tr>
<td>IX</td>
<td>&gt; 700</td>
<td>1 % DDM, 1 % digitonin, 0.2 % Triton</td>
<td>10 (a - k)</td>
</tr>
<tr>
<td>X</td>
<td>&gt; 700</td>
<td>1 % digitonin</td>
<td>6 (a - f)</td>
</tr>
</tbody>
</table>

Peroxisomal membranes were treated with 1 % (w/v) DDM, 0.2 % (v/v) Triton X-100 or 1 % (w/v) digitonin to solubilize membrane proteins. After BN-PAGE several protein complexes (Complex I to X) of different sizes (150 - >700 kDa) could be detected. Second dimension analysis using SDS-PAGE revealed that Complex VIII, IX and X dissociated into their components. For the other protein complexes, the second dimension analysis was not clear and thus is not mentioned in this table.

Pex3p during the solubilization with 1 % (v/v) Triton X-100 (Fig. 2B). Upon solubilization with 1 % (w/v) digitonin, 3 protein bands were visible after BN-PAGE: VII, IX and X.

The minor amounts of the matrix proteins AO and DHAS, which was present in the membrane pellet fraction fraction (Fig. 2A), were detected using Western blotting as approximately 600 kDa (octameric AO) and 150 kDa (dimeric DHAS) protein complexes (data not shown) after BN-PAGE, but their protein bands were not visible after silver staining of the BN-PAGE gel (Fig. 3). The amount of the matrix proteins is probably too low to be visible as a clear distinct protein band.

In order to analyze whether the protein bands observed in the silver stained BN-PAGE gel represented protein complexes, a second dimension denaturing gel electrophoresis (SDS-PAGE) step was performed. Therefore, lanes from the BN-PAGE gel were layered on top of a SDS-PAGE gel. After electrophoresis and silver staining (Fig. 4), several of the protein bands observed with BN-PAGE clearly separated in a number of proteins (see also Table I). For example, analysis of the second dimension gels revealed that protein band VIII, which was obtained after treatment of the peroxisomal membranes with 0.2 % (v/v) Triton X-100, was composed of several proteins (a - h). Moreover, apparently the same proteins were also observed in the second dimension gel prepared from 1 % (w/v) DDM solubilized samples (Fig. 5). Although protein band VIII was not readily resolved with BN-PAGE after solubilization with 1 % (w/v) DDM (Fig. 3), the proteins of this complex could clearly be observed after analysis of the second dimension SDS-PAGE.

A different protein band, IX dissociated in at least 10 proteins (a - k) after a second dimension SDS-PAGE. This complex and its protein components were observed after
Protein complexes in the peroxisomal membrane

Fig. 6. Protein complex containing the peroxisomal membrane protein Pex14p. Western blot analysis of a BN-PAGE gel using specific antibodies against Pex14p demonstrated that this protein is present in a larger complex. This complex could be solubilized from peroxisomal membranes using 1 % (w/v) DDM, 0.2 % (v/v) Triton X-100 or 1 % (w/v) digitonin. The size of this complex is estimated at 400-500 kDa. The locations of the protein bands (IV - X) observed in Fig. 3 are indicated.

solubilization with all detergents used (Fig. 5), implying that this complex is quite stable under these conditions. Another protein band, X, was observed only after solubilization of the membranes with 1 % (w/v) digitonin. This protein complex consisted of approximately 6 proteins (a - f). After a closer examination (Fig. 5), protein band X most probably contains the same proteins as protein band VIII despite the fact that X is more than 100 kDa larger than VIII. In the second dimension gel additional proteins have not been observed in protein band X, which suggest that it could consist of multiple copies of VIII.

In the second dimension gels, for some protein bands (e.g. I, II, and VI) only a few distinct proteins could be detected, while for other protein bands (III, IV, and V) no distinct proteins could be observed at all. Remarkably, protein band VII could be observed as a sharp distinct band in the BN-PAGE gel (Fig. 3). Analysis of the second dimension gels indicated that this protein complex is actually an homo-oligomer, since in the second dimension of this protein complex only one protein with a molecular weight of approximately 60 kDa was detectable (Fig. 4).

**Pex14p is present in an approximately 400-500 kDa protein complex**

The proteins, obtained after solubilization of peroxisomal membranes with 1 % (w/v) DDM, 0.2 % (v/v) Triton or 1 % (w/v) digitonin, in a BN-PAGE gel were transferred to a PVDF-membrane and decorated with specific antibodies against the peroxisomal membrane proteins Pex3p and Pex14p. Unexpectedly, although Pex3p was solubilized under these conditions (Fig. 2B), this protein could not be detected on the blot after BN-PAGE. This could be caused by several reasons, like for instance an inability of the protein to enter the BN-PAGE gel, to become transferred to the membrane, or degradation of Pex3p. In contrast, Pex14p was clearly detectable as a protein band of approximately 400-500 kDa after solubilization with 1 % (w/v) DDM,
Fig. 7. Second dimension analysis of the protein complex containing Pex14p using SDS-PAGE. Protein complexes solubilized with 0.2 % (v/v) Triton X-100 were subjected to BN-PAGE. Slices, corresponding to the size of the complex containing Pex14p, were cut out from BN-PAGE gels and were layered on top of a SDS-PAGE gel. After electrophoresis, part of the gel was stained with silver (lane 2), and another part subjected to Western blotting (lane 3). Lane 1 of the SDS-PAGE gel contains the marker proteins.

0.2 % (v/v) Triton X-100 or 1 % (w/v) digitonin, suggesting that this peroxin (molecular weight is 42 kDa) is present in a larger protein complex (Fig. 6). The position of this protein complex on the Western blot is between protein band VII and VIII. However, in this region of a silver stained BN-PAGE gel (Fig. 3), a clear distinct protein band, what could represent the Pex14p-containing protein complex, was not observed. Apparently, the amount of the Pex14p-containing protein complex was too low to be detected with silver staining, or the protein band was too diffuse to be recognized as a clear distinct band.

Since the complex containing Pex14p was most dominant upon solubilization with 0.2 % (v/v) Triton X-100, this complex was further analyzed by a second dimension denaturing SDS-PAGE. Gel slices, corresponding to the complex containing Pex14p (above the distinct protein band VII), were cut out from BN-PAGE gels loaded with 0.2 % (v/v) Triton X-100 solubilized membranes. These slices were layered on top of a SDS-PAGE gel. After electrophoresis, part of the gel was stained with silver, and another part subjected to Western blotting. As is evident from the silver staining, approximately 8 dominant and several minor protein bands could be observed (Fig. 7). The protein constitution of this protein complex differs from the protein complexes VIII, X and IX (fig. 5). It remains uncertain whether all these proteins are actual components of the same complex as Pex14p, or originate from complexes that overlap with the Pex14p containing complex in the BN-PAGE gel.

Western blot analysis using specific antibodies against Pex14p (Fig. 7), revealed that Pex14p could be recovered from the BN-PAGE gel as a monomer (ca. 42 kDa). Besides the monomeric protein band, also lower specific bands are present, which most likely reflect degradation products of Pex14p. Apparently, these Pex14p degradation products were initially present in the protein complex, or Pex14p is
partially degraded during the second dimension electrophoresis process.

**Discussion**

In this paper we provide evidence for the existence of protein complexes in peroxisomal membranes of *H. polymorpha*, using BN-PAGE to separate the solubilized protein complexes from purified peroxisomal membrane samples. We demonstrated that at least 10 protein complexes could be distinguished dependent on the choice and concentration of the detergent. These protein complexes differed in size (ranging from 150 kDa to over 700 kDa) and composition, as became evident from analyses of second dimension SDS-PAGE gels.

The presence of protein complexes in peroxisomal membranes has already been suggested from genetic (two-hybrid system) and biochemical (immunoprecipitation, ligand blots) studies, which pointed to specific interactions between various peroxins. Furthermore, it has been demonstrated that the peroxisomal membrane protein Pex11p is present in mature organelles as dimers [8], and the peroxisomal ABC transporters could form homo- and heterodimers [178].

A peroxisomal protein, which has been intensively studied in this respect, is the peroxin Pex14p. Pex14p is a membrane-associated protein [41, 42, 47] and a constituent of the peroxisomal matrix protein import machinery. Pex14p is thought to function together with two other peroxins, Pex13p and Pex17p, as a docking site on the peroxisomal membrane for the cytosolic PTS receptors Pex5p and Pex7p. This was concluded from the observations that Pex13p and Pex14p contain cytosolically exposed domains that can physically interact with the PTS1 receptor Pex5p [29, 30, 41, 147] and the PTS2 receptor Pex7p [41, 46]. Moreover, interactions between Pex13p and Pex14p [41] and Pex14p and Pex17p [44] have been demonstrated. Using BN-PAGE we found that *H. polymorpha* Pex14p was present in a protein complex of approximately 400-500 kDa. In addition, second dimension analysis demonstrated that more proteins are probably part of the Pex14p-containing protein complex (Fig. 7), but characterization of these proteins has to be realized yet. Proteins, which could possibly be expected as constituents of this complex are Pex13p and Pex17p, based on the demonstrated interactions between these proteins. Unfortunately, as a result of the absence of specific antibodies against these proteins in *H. polymorpha* this can not be examined. Possible novel components of this complex can be identified by microsequencing.

Potential complications of BN-PAGE as a mode to characterize protein complexes are the effect of the selected detergent and the detergent/protein ratio on the stability of protein complexes and proteins. This has been clearly demonstrated by the solubilization of Pex3p (Fig. 2B): treatment of peroxisomal membranes with Triton X-100 resulted in reduction of Pex3p levels, especially at higher detergent concentrations. Also in other systems this effect has been observed. For example, the composition of the yeast mitochondrial F1F0-ATP synthase complex seemed to be affected by the detergent/protein levels. At low Triton X-100 concentrations F1F0-ATP synthase was a dimer, while at higher concentrations only the monomeric form was detected [188]. Moreover, in this way three dimer-specific subunits were identified. In addition, Schägger *et al.* [189] recently demonstrated the presence of supercomplexes in the respiratory chains of yeast and mammalian mitochondria. These complexes were stable after solubilization with digitonin (even at increasing digitonin/protein ratios) and low concentrations of Triton X-100, but solubilization with DDM resulted in the dissociation into two smaller complexes.

The data presented in this work demonstrated that BN-PAGE is a powerful tool for detection and analysis of protein
complexes in peroxisomal membranes. Further examination of protein complexes by BN-PAGE will allow the characterization of interacting proteins, using known peroxins as markers. This technique will open new territories in the study of peroxisome biogenesis, and will be useful to elucidate the still unknown peroxisomal protein translocation machinery.