Physical Interactions of the Peroxisomal Targeting Signal 1 Receptor Pex5p, Studied by Fluorescence Correlation Spectroscopy*

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We have studied Hansenula polymorpha Pex5p and Pex8p using fluorescence correlation spectroscopy (FCS). Pex5p is the Peroxisomal Targeting Signal 1 (PTS1) receptor and Pex8p is an intraperoxisomal protein. Both proteins are essential for PTS1 matrix protein import and have been shown to physically interact. We used FCS to analyze the molecular role of this interaction. FCS is a very sensitive technique that allows analysis of dynamic processes of fluorescently marked molecules at equilibrium in a very tiny volume. We used this technique to determine the oligomeric state of both peroxins and to analyze binding of Pex5p to PTS1 peptides and Pex8p. HpPex5p and HpPex8p were overproduced in Escherichia coli, purified by affinity chromatography, and, when required, labeled with the fluorescent dye Alexa Fluor 488. FCS measurements revealed that the oligomeric state of HpPex5p varied, ranging from monomers at slightly acidic pH to tetramers at neutral pH. HpPex5p formed monomers at all pH values tested. Using fluorescein-labeled PTS1 peptide and unlabeled HpPex5p, we established that PTS1 peptide only bound to tetrameric HpPex5p. Upon addition of HpPex8p, a heterodimeric complex was formed consisting of one HpPex5p and one HpPex5p molecule. This process was paralleled by dissociation of PTS1 peptide from HpPex5p, indicating that Pex5p may play an important role in cargo release from the PTS1 receptor. Our data show that FCS is a powerful technique to explore dynamic physical interactions that occur between peroxins during peroxisomal matrix protein import.

Peroxisomes are single membrane-bound organelles that are present in most aerobic eukaryotic cells. They acquire their matrix proteins by uptake of newly synthesized proteins from the cytosol. This process occurs predominantly via two pathways that rely on either of two conserved peroxisomal targeting signals (PTS1 and PTS2). The vast majority of matrix proteins contain a PTS1 that consists of a tripeptide motif (SKL or variants thereof) located at the extreme C terminus of the protein. PEX genes are essential for peroxisome biogenesis and have been numbered 1 to 29 according to the order of description (extensively reviewed in Ref. 1). Most of them encode proteins, designated peroxins, that play a role in peroxisomal matrix protein import.

The PEX5 gene encodes the PTS1 receptor, Pex5p, which binds newly synthesized PTS1 proteins in the cytosol. This receptor is proposed to be translocated together with its cargo into the peroxisomal matrix (2). Dammai and Subramani (3) presented compelling evidence that human Pex5p, indeed, cycles between these two subcellular compartments.

Pex5p is the only known intraperoxisomal peroxin involved in matrix protein import. It is not required for association of Pex5p at the receptor docking complex on the peroxisomal membrane (4) but is important at a later stage in the import process. Recently, Agne et al. (5) showed that ScPex8p is important for the association of the receptor docking complex (comprising Pex14p and Pex17p) with another membrane-bound complex that contains the ring finger proteins Pex2p, Pex10p, Pex12p. Both membrane-bound protein complexes are involved in matrix protein import and possibly only function upon connection by Pex8p at the trans-site of the peroxisomal membrane (5).

Results of two-hybrid analysis and co-immune precipitations in Saccharomyces cerevisiae revealed that ScPex8p also physically interacts with ScPex5p in the organelar matrix (4). The molecular role of this interaction is still unknown. Most known Pex8 proteins contain a PTS1. However, the PTS1 of Pex8p is not the sole potential site of interaction with Pex5p, because this interaction is still robust when the PTS1 is removed (4). Most likely, the PTS1 of Pex8p is involved in targeting of the protein to peroxisomes instead of being important for its ultimate role in PTS1 matrix protein import.

To understand the molecular role of the physical interaction between Pex5p and Pex8p in matrix protein import, we studied these peroxins using a novel approach in peroxisome research, namely fluorescence correlation spectroscopy (FCS). FCS is a very sensitive technique developed for the study of dynamic processes of fluorescently labeled molecules at equilibrium in a tiny “volume” (6–8). FCS was developed in the mid-1970s but has only recently been recognized as a powerful tool in various areas of research. In FCS measurements, fluorescence intensity fluctuations are recorded only from those molecules that diffuse through an open volume element created by a focused laser beam. The time required for passage of molecules through this volume element is determined by the diffusion time, which is inversely proportional to the diffusion coefficient that is related to the size of the molecule. Thus, diffusion constants obtained from the analysis of fluorescence intensity fluctuations allow differentiation between relatively small, rapidly diffusing molecules and larger, more slowly diffusing ones.

Advantages of FCS over the techniques that are generally used to study protein-protein interactions (yeast two-hybrid analysis, GST pull-down experiments, co-immune precipitations) is that the size of protein complexes can be estimated.
allowing prediction of the stoichiometry of the interacting components. In addition, the fraction of free and bound molecules at equilibrium can be determined. Moreover, the proteins to be studied are not immobilized and the measurements are performed at relatively low protein concentrations (nm range), which reduces the risk of nonspecific aggregation.

In this study we analyze the interactions between purified HpPex8p (polymerase Pex5p (9) and Pex8p (10) and synthetic PTS1 peptides in vitro. In each experiment one of these components was fluorescently labeled and analyzed by FCS. Calculation of the diffusion constants of the fluorescent molecule allowed us to determine the oligomeric state of the individual proteins and the physical interactions between these molecules. Our studies indicate that FCS is a powerful technique to explore the dynamics of physical interactions that occur during peroxisomal matrix protein import.

EXPERIMENTAL PROCEDURES

Peptides and Proteins—Peptides PTS1 (ASKL-COOH) and FI-PTS1 (ASSASKL-COOH) covalently bound to fluorescein (Fl) at the N termini (59) were purchased from Eurosequence (Groningen, the Netherlands). The concentration of Fl-PTS1 was determined spectrophotometrically using a linear extinction coefficient $e_{1%}$ at 488 nm of 7,8 $\times$ 10$^4$ M$^{-1}$ cm$^{-1}$.

For HpPex5p purification, a carboxyl-terminal His$_6$-tagged version was overproduced in E. coli. A PEX5 PCR product, obtained using primers 5'-GGCCCATGCGATTTGAGGAGCG-3' and 5'-GGCACTCAGTTGAGGAGCG-3' was cloned as a 1.7-kb NcoI-BglII fragment (sites introduced by PCR) into vector pQE-60 (Qiagen, Hilden, Germany). The resulting plasmid was designated pQE60-PEX5-HIS6.

For overproduction of carboxyl-terminal His$_6$-tagged HpPex8p, a PEX8-HIS6 PCR product was obtained using primers 5'-CTGACTCTC-3' and 5'-CCGAGATCTTACGTTAGTTTTGCG-3' and cloned into vector pQE-70. The resulting plasmid was designated pQE70-PEX8-HIS6.

To this purpose the PEX5-HIS6 (blunted $\frac{1}{3}$ fragment from the pQE60-PEX5-HIS6) and pQE70-PEX8-HIS6 were transformed to E. coli M15 (pREP4). Transforms were grown as detailed in the Qiagen expression system manual. Overproduction was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside and incubation for 3 h at 30 °C. All subsequent steps were performed at 4 °C. Cells were harvested by centrifugation and resuspended in 50 mM phosphate buffer, pH 7.0, containing 300 mM NaCl, 1% Tween 20, 10% glycerol, 0.2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, complete T7 protease inhibitor (Roche Applied Science), 50 μg/ml RNase, and 100 μg/ml DNase and subsequently broken using a French Press. Cell debris and other insoluble material were removed by centrifugation (10,000 × g, 20 min). Supernatants were incubated for 1 h with nickel-nitrilotriacetic acid resin (500 mg protein/ml resin; Qiagen) followed by extensive washing with buffer A (50 mM phosphate buffer, pH 7.0, containing 100 mM NaCl) with increasing concentrations of imidazole (up to 40 mM), followed by elution with buffer A containing 200 mM imidazole. Pex5p or Pex8p peak fractions, determined by Western blotting using anti-Pex5p or anti-Pex8p antibodies, were further purified by anion exchange chromatography (MonoQ; Amersham Biosciences) using a linear gradient of 0.1–1 M NaCl in 20 mM Bis-Tris- HCl buffer (pH 7.0) for HpPex5p and in 20 mM Tris-HCl buffer (pH 7.8) for HpPex8p. HpPex5p was further purified by gel filtration chromatography (Superose 12; Amersham Biosciences) in buffer A containing 300 mM NaCl. HpPex5p and HpPex8p were labeled with Alexa Fluor 488 using the protein labeling kit of Molecular Probes (Leiden, The Netherlands) and dialyzed against buffer prior to the FCS measurements. Dialysis did not prevent the presence of residual free Alexa Fluor 488 in the samples.

Analysis of the Functionality of HpPex5p–His$_6$ and HpPex8p–His$_6$—The functionality of carboxyl-terminal His$_6$-tagged HpPex5p was tested by introducing the PEX5-HIS6 gene under control of the TAP (transactivation) promoter (P_TAP) in a H. polymorpha PEX5 deletion strain (Δpex5) (9). To this purpose the PEX5-HIS6 cassette was isolated as a 1.8-kb NcoI (blunted) HindIII fragment from the pQE60-PEX5-HIS6 plasmid and ligated into the shuttle vector pH55 together with a 0.5-kb BamHI (blunted SacI) PEX5 promoter fragment (BamHI site introduced by PCR). The resulting plasmid was transformed H. polymorpha Δpex5. The functionality of carboxyl-terminal His$_6$-tagged HpPex8p was determined by integrating the carboxyl-terminal part of the HpPEX8-HIS6 gene of pQE70-PEX8-HIS6 in H. polymorpha NCYC495 leu 1.1 in such a way that only HpPex8p–His$_6$ is produced. To this purpose the carboxyl-terminal part of PEX8-HIS6 was isolated as a 1.3-kb HindIII fragment from pQE70-PEX8-HIS6 and ligated in vector pH55 digested with HindIII. The resulting plasmid was linearized with MluI and transformed to H. polymorpha NCYC495 leu 1.1. Prototrophic transformants were selected. The synthesis of His$_6$-tagged HpPex5p and His$_6$-tagged HpPex8p in the two constructed strains was determined by Western blot analysis using monoclonal antibodies against the histidine tag (Dianova GmbH, Hamburg, Germany). In addition, these strains were tested for their ability to grow on methanol, which is indicative for normal peroxisome formation (9, 10).

FCS—Protein concentrations were calculated from the absorption at 280 nm, using molar extinction coefficients of 5.8 × 10$^4$ M$^{-1}$ cm$^{-1}$ for HpPex8p and 9.3 × 10$^4$ M$^{-1}$ cm$^{-1}$ for HpPex8p calculated from their aromatic amino acid content. For binding studies, samples were preincubated at 37 °C for 1 h prior to the measurements.

A ConfoCor 1 FCS setup (Zeiss, Gottingen, Germany) (8, 11), equipped with an argon ion laser line of 488 nm and a 40 × 1.2 water immersion apochromat objective, was used in all experiments. The light intensity was adjusted by using various neutral density filters. As a sample chamber, a 96-well chamber was used. The pinhole was adjusted to 40 μm; the alignment and focusing of the setup were frequently checked by measuring the photon count traces of 10 nm Rhodamine 110. Autocorrelation traces were acquired during 30 or 60 s and repeated 10 times. All the experiments were conducted at room temperature. The autocorrelation curves were globally analyzed to a model consisting of diffusion and triplet state (12) as shown in Equation 1.

\[
G(r) = 1 + \frac{1}{N} \left( \frac{1 - T + T e^{-r^2/\tau_{j}}} \tau_{j} \right) \sum_{i} \alpha_{i} \left( \frac{1}{\tau_{d,i}} \right) \left( \frac{1 + \left( \frac{r}{\tau_{j}} \right)}{\left( \frac{r}{\tau_{j}} \right)^2} \right) \left( \frac{1}{\alpha_{i}} \right)
\]

where \(N\) is the average number of molecules, \(T\) is the fraction of molecules in the triplet state, and \(1/T_{j}\) is the corresponding triplet decay rate, \(\tau_{j}\) is the diffusion time of molecular species \(j\), \(\omega_{i}\) and \(\omega_{j}\) are the equatorial and axial radii of the confocal volume set up by the laser beam, and \(\omega_{i} \omega_{j}\) is the structural parameter. Equation 1 is written in a multicomponent form with \(\omega\) the fraction of molecular species \(i\). In Equation 1 it is assumed that all species have the same triplet characteristics and molecular brightness. The dimensions of the confocal detection volume and the structural parameter are obtained from global analysis of the autocorrelation curves of the standard Rhodamine 110 in water having a diffusion constant of 280 μm$^2$s$^{-1}$ (13).

In global analysis several correlation functions are combined in one data set and simultaneously fitted with certain parameters linked over the set, ensuring more reliable parameter recovery and thus more consistent representation of the physical processes under investigation (14). All the experimental autocorrelation curves were globally fitted by the theoretical autocorrelation function given in Equation 1 using the FCS data processor 1.3 of the Scientific Software Technologies Center of Belarusian State University, Belarus. The quality of the fits and performance of the optimization methods was improved by fixing the parameters that are known a priori from independent experiments (for instance, free Alexa Fluor 488).

The relation between diffusion time and diffusion constant is shown in Equation 2.

\[
D^{-1} = \frac{6 \pi \eta \delta}{kT}
\]
A diffusion model was used, where the diffusion time of free Alexa Fluor 488 was fixed to the earlier estimated value. A rigorous error analysis at a confidence interval of 67% for the diffusion time of HpPex5p was performed (14). The diffusion constant of HpPex5p at pH 7.2 (see Fig. 1A) appeared to be 38 \( \mu m^2s^{-1} \) (with confidence limits 36–41 \( \mu m^2s^{-1} \)). This diffusion constant corresponds to a molecular mass of ~250 kDa (198–312 kDa), assuming that the protein has a globular shape (see Equation 4 under “Experimental Procedures”). Because the molecular mass of HpPex5p calculated from its amino acid sequence amounts to 63.9 kDa (9), we conclude that the protein formed tetramers under the prevailing experimental conditions. At pH 6.0 (Fig. 1B), the diffusion constant of HpPex5p increased to a value that corresponds to a molecular mass of 61 kDa (48–83 kDa), indicative of a monomeric conformation. These data indicate that the oligomeric state of HpPex5p is dependent on the pH and varies within a relatively small pH interval.

**PTS1 Peptides Predominantly Bind to Tetrameric HpPex5p**

In order to study binding of a peptide containing the consensus sequence (SKL) of the PTS1, a chemically synthesized peptide (ASSASKL) was labeled with fluorescein (Fl-PTS1). Autocorrelation curves in the presence and absence of HpPex5p were collected. These experiments were conducted at two different pH values. In Fig. 2A three autocorrelation curves (of ten) are shown together with fitted curves and residuals for Fl-PTS1 at pH 7.2. A single diffusion constant of 224 \( \mu m^2s^{-1} \) (206–239 \( \mu m^2s^{-1} \)) was obtained at pH 7.2 (Fig. 2A). At pH 6.0, a similar value was obtained (data not shown). In Fig. 2B the same set of curves is presented for Fl-PTS1 in the presence of HpPex5p at pH 7.2. The best fit was obtained when a two-component diffusion model was used in which the diffusion time of free Fl-PTS1 was fixed in the analysis. In the presence of HpPex5p the autocorrelation curve shifted to longer diffusion times, indicating binding of the peptide to a larger molecule (Fig. 2B legend and Table 1). At the experimental conditions used, the two-component diffusion model revealed that 14% of the peptide was bound to HpPex5p. The diffusion constant of the bound PTS1 peptide corresponded to a molecular mass of ~277 kDa, indicating that it was bound to tetrameric HpPex5p. In an identical experiment performed at pH 6.0 (data not shown), only the short diffusion time corresponding to free Fl-PTS1 was observed, and no additional diffusion time could be distinguished. This indicates that no significant binding of the PTS1 peptide to HpPex5p occurs at this pH.

In control experiments performed at both pH values using purified HpPex8p (see below), only the short diffusion time of the free peptide could be recovered. Hence, the PTS1 peptide specifically binds to HpPex5p but not to HpPex8p.

**HpPex5p Interacts with HpPex8p**

So far, no data have been reported on the oligomeric state of Pex8p. To facilitate purification, a carboxyl-terminal His\(_6\)-tagged version of *H. polymorpha* Pex5p (HpPex5p) was overproduced in *E. coli*. The His\(_6\)-tagged HpPex5p was fully functional because synthesis of this protein in a *H. polymorpha* PEX5 deletion strain fully restored the capacity of the cells to grow on methanol (data not shown).

Purified HpPex5p was labeled with the fluorescent dye Alexa Fluor 488, which enabled us to follow the fate of the protein in solution using FCS. In Fig. 1 examples of three autocorrelation curves (of ten) are shown together with fitted curves and residuals. The autocorrelation curves were fitted globally to a diffusion model including triplet kinetics (see “Experimental Procedures”). The best fit was achieved when a two-component
Interactions of Pex5p

The concentration of Alexa Fluor 488-labeled HpPex8p was 50 nM, the HpPex5p concentration was 500 nM, and the PTS1 peptide concentration was 5 μM.

<table>
<thead>
<tr>
<th>pH</th>
<th>Proteins</th>
<th>PTS1 peptide</th>
<th>Diffusion constant</th>
<th>Calculated molecular mass</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>μm²s⁻¹</td>
<td>kDa</td>
</tr>
<tr>
<td>6.0</td>
<td>HpPex8p</td>
<td>–</td>
<td>58 (53–64)</td>
<td>73 (54–95)</td>
</tr>
<tr>
<td>6.0</td>
<td>HpPex8p + HpPex5p</td>
<td>45 (42–49)</td>
<td>157 (122–197)</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>HpPex5p + HpPex8p</td>
<td>+ 47 (46–50)</td>
<td>135 (118–152)</td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>HpPex8p</td>
<td>–</td>
<td>61 (58–65)</td>
<td>63 (52–73)</td>
</tr>
<tr>
<td>7.2</td>
<td>HpPex8p + HpPex5p</td>
<td>49 (46–51)</td>
<td>127 (106–149)</td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>HpPex8p + HpPex5p</td>
<td>+ 45 (42–49)</td>
<td>158 (124–196)</td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>HpPex8p</td>
<td>–</td>
<td>70 (65–76)</td>
<td>60 (47–75)</td>
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<tr>
<td>8.0</td>
<td>HpPex8p + HpPex5p</td>
<td>51 (47–56)</td>
<td>154 (121–183)</td>
<td></td>
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<tr>
<td>8.0</td>
<td>HpPex8p + HpPex5p</td>
<td>+ 53 (48–59)</td>
<td>139 (100–185)</td>
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</table>

The measurements were performed at pH 7.2. The concentration of Alexa Fluor 488 dye, a diffusion constant of ~49 μm²s⁻¹ was obtained (Table II). This value corresponds to a molecular mass of ~127 kDa and suggests the formation of heterodimeric complexes of HpPex8p and HpPex5p. Under these conditions (i.e., a 10-fold molar excess of HpPex5p), most HpPex8p was bound to HpPex5p. Similar results were obtained when the experiments were performed at pH 6.0 or 8.0 (Table II).

Upon addition of excess (500 nM) unlabeled HpPex8p to the mixture, the diffusion constant increased again (data not shown), indicating that indeed the high molecular mass species represented dimers consisting of labeled HpPex8p and unlabeled HpPex5p. Addition of labeled HpPex8p (50 nM) to a mixture of HpPex5p (500 nM) and PTS1 peptide (5 μM) resulted in a similar autocorrelation curve, indicating that the presence of PTS1 peptide did not influence the HpPex5p-HpPex8p interaction (Table II).

HpPex5p Causes Dissociation of the HpPex5p-PTS1 Peptide Complex—Next we tested whether HpPex8p affected binding of PTS1 peptide to HpPex5p. To test this, unlabeled HpPex8p (50 nM) was added to a mixture of unlabeled HpPex5p (10 nM) and fluorescein-labeled PTS1 peptide (5 μM) preincubated at 37°C for 1 h. The experimental autocorrelation curves were best fitted with a two-component diffusion model, corresponding to free peptide and peptide bound to tetrameric HpPex5p. No significant amounts of the FI-PTS1 peptide bound to HpPex5p-HpPex8p heterodimers could be detected. The addition of HpPex8p resulted in a decrease in the fraction of PTS1 peptide bound to tetrameric HpPex5p. At the experimental conditions used, the fraction of bound peptide decreased to 9% (compare with 14% that was bound in the absence of HpPex8p; Table I).

DISCUSSION

In this study we used FCS to study physical interactions of two peroxins, Pex5p and Pex8p, involved in peroxisomal matrix protein import. A wealth of experimental data has been reported showing peroxin-peroxin and receptor-PTS interactions (reviewed in Ref. 1). However, the techniques used (e.g., yeast two-hybrid analysis, co-immune precipitation, surface plasmon resonance analysis) do not allow determination of the stoichiometry or kinetics of these interactions. Here we show that FCS is a powerful tool to assess the dynamics of peroxin interactions. FCS is generally used to study binding of small ligands to relatively large structures (proteins, membranes). We show that FCS is also suitable to study the interactions of proteins of comparable molecular mass (e.g. HpPex5p and HpPex8p) when global analysis of many experimental data is applied. A major advantage of FCS is the use of low protein concentrations, which prevents the possible formation of nonspecific protein aggregates. Pex5p has been predicted to function as a cycling

FIG. 2. Examples of experimental autocorrelation curve (dots), fitted (solid line), and residuals (upper inset) of FI-PTS1 in the absence (A) or presence (B) of HpPex5p at pH 7.2. The concentration of FI-PTS1 was 50 nM. HpPex5p was used at a concentration of 10 nM. After global analysis of 10 experimental curves to the model of Equation 1, the following parameters were obtained. A, one-component fit: τ = 3.1 μs, T = 10%; FI-PTS1-free τ = 80 μs (75–86 μs). B, two-component fit: τ = 1.7 μs, T = 10%; FI-PTS1-free τ = 80 μs (fixed); HpPex5p τ = 479 μs (424–521 μs).

Table I

<table>
<thead>
<tr>
<th>Diffusion constant</th>
<th>Calculated molecular mass</th>
<th>Fraction of PTS1 peptide bound to HpPex5p</th>
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<tr>
<td>μm²s⁻¹</td>
<td>kDa</td>
<td>%</td>
</tr>
<tr>
<td>FI-PTS1</td>
<td>224 (206–239)</td>
<td>1.1 (1.06–65)</td>
</tr>
<tr>
<td>FI-PTS1 + HpPex8p</td>
<td>37 (34–42)</td>
<td>277 (202–375)</td>
</tr>
<tr>
<td>FI-PTS1 + HpPex8p</td>
<td>37 (fixed)</td>
<td>277 (202–375)</td>
</tr>
<tr>
<td>HpPex8p</td>
<td></td>
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</table>

8.0)-73 kDa (pH 6), which is similar to the mass calculated from its amino acid composition (77 kDa) (10). We therefore conclude that purified HpPex8p occurs as a monomer independent of the pH.

Upon addition of excess unlabeled HpPex5p to the Alexa Fluor 488-labeled HpPex8p solution (at pH 7.2), again a good two-component fit was obtained. After fixing the diffusion time of free Alexa Fluor 488 dye, a diffusion constant of ~49 μm²s⁻¹ was obtained (Table II). This value corresponds to a molecular mass of ~127 kDa and suggests the formation of heterodimeric complexes of HpPex8p and HpPex5p. Under these conditions (i.e., a 10-fold molar excess of HpPex5p), most HpPex8p was bound to HpPex5p. Similar results were obtained when the experiments were performed at pH 6.0 or 8.0 (Table II).

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receptor between the cytosol and the peroxisomal matrix. The carboxyl-terminal domain of HpPex5p contains tetratricopeptide repeats that are responsible for binding PTS1 signals, whereas the amino terminus interacts with several other peroxins (e.g. Pex13p, Pex14p, Pex12p). This part of the protein is also involved in Pex5p oligomerization (15).

In this study we showed that HpPex5p exists in different oligomeric conformations that vary with the pH. HpPex5p predominantly formed monomers at pH 6.0, whereas at pH 7.2 it is tetrameric. This observation may add to an explanation of the apparent conflicting data reported for mammalian Pex5p. Using gel filtration chromatography at pH 8.0, human Pex5p was found to be tetrameric (15), whereas for rat and Chinese hamster Pex5p it was concluded, based on similar experiments performed at pH 7.2 (17), that the protein formed dimers.

Only at pH 7.2 did PTS1 peptides bind to HpPex5p, indicating that ligand binding may be confined to tetramers. This finding is in line with the recently proposed “pre-implex” model for peroxisomal matrix protein import, which predicts that peroxisomal matrix protein import is mediated by tetrameric Pex5p molecules (18). The pre-implex model proposes that one Pex5p tetramer binds four PTS1 proteins in the cytosol. Because peroxisomal matrix proteins are often imported as oligomers that contain one PTS1 per subunit, very large protein complexes (the so-called pre-implexes) containing several Pex5p tetramers and different oligomeric PTS1 proteins may be formed prior to import (18).

Analysis of Pex5p-PTS1 binding revealed that at pH 7.2 14% of the peptide was bound to HpPex5p under the experimental conditions used. This corresponds to an equilibrium dissociation constant of \(-18\) nM, which is in the same range as reported for the carboxyl-terminal tetratricopeptide domain of human Pex5p, based on fluorescence anisotropy measurements (equilibrium dissociation constant 35–70 nM (19, 20)).

The apparent small fraction of bound peptide (14%) obtained under the experimental conditions used is because of the low protein and peptide concentrations (in the nanomolar range). In all measurements described in this study, the fluorescent molecule to be analyzed was present at a final concentration of 50 nM, which is suitable for FCS experiments. In FCS, the focused laser beam illuminates a very tiny volume element (\(-1\) \(\mu\)m\(^3\)). To detect fluctuations in fluorescence due to diffusion of fluorescent molecules into and out of this volume element, it should contain, on average, not more than one fluorescent molecule, which is reached at concentrations in the nanomolar range (21).

It is likely that the proteins analyzed in this study are also functioning at low concentrations under physiological conditions. Because PTS1 protein import is very efficient, newly synthesized PTS1 proteins are generally not detectable in the cytosol. Also, HpPex5p is not an abundant component of the cytosol, and in peroxisomes both HpPex5p and HpPex8p are present at relatively low concentrations (9, 10).

Our results indicated that HpPex5p is monomeric. Using FCS, we could establish a physical interaction between HpPex5p and HpPex8p, namely the formation of heterodimers. The Pex5p-Pex8p interaction was previously reported for the bakers’ yeast orthologs using two-hybrid analysis and co-immune precipitations (4). As for bakers’ yeast, the interaction between HpPex5p and HpPex8p was not the result of binding of the PTS1 of HpPex5p to the tetratricopeptide domain of HpPex5p, because a His\(_8\) tag was added to the carboxyl terminus of HpPex8p, thereby destroying its PTS1.

When HpPex8p was added to a solution of tetrameric HpPex5p that had bound PTS1 peptide, the fraction of bound PTS1 peptide significantly decreased. At the same time

![Hypothetical model of Pex5p and Pex8p interactions during one cycle of PTS1 protein import](image)
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REFERENCES