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Fluorescence analysis of the *Hansenula polymorpha* peroxisomal targeting signal-1 receptor, Pex5p

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Correct sorting of newly synthesized peroxisomal matrix proteins is dependent on a peroxisomal targeting signal (PTS). So far two PTSs are known. PTS1 consists of a tripeptide that is located at the extreme C terminus of matrix proteins and is specifically recognized by the PTS1-receptor Pex5p. We studied *Hansenula polymorpha* Pex5p (HpPex5p) using fluorescence spectroscopy. The intensity of Trp fluorescence of purified HpPex5p increased by 25% upon shifting the pH from pH 6.0 to pH 7.2. Together with the results of fluorescence quenching by acrylamide, these data suggest that the conformation of HpPex5p differs at these two pH values. Fluorescence anisotropy decay measurements revealed that the pH affected the oligomeric state of HpPex5p, possibly from monomers/dimers at pH 6.0 to larger oligomeric forms at pH 7.2. Addition of dansylated peptides containing a PTS1, caused some shortening of the average fluorescence lifetime of the Trp residues, which was most pronounced at pH 7.2. Our data are discussed in relation to a molecular model of HpPex5p based on the three-dimensional structure of human Pex5p.

Keywords: peroxisome; Pex5p; protein targeting; PTS1; Trp-fluorescence.

Eukaryotic cells are characterized by compartmentation of specific functions in highly specialized cell organelles. Most organellar proteins are encoded by nuclear genes and synthesized by cytosolic ribosomes. In order to ensure that these proteins reach the correct destination in the cell, they contain sorting signals that are recognized by specific receptors, which guide them to the proper protein translocation machinery.

Compared to other cell organelles, relatively little is known of targeting and import of peroxisomal proteins. Currently, two peroxisomal targeting signals (PTS) have been identified (designated PTS1 and PTS2) that are necessary and sufficient to target peroxisomal matrix proteins to the correct organelle [1]. The PTS1 is the most common signal, consisting of a tripeptide located at the extreme C terminus of the protein. The consensus sequence is SKL, but various conserved variants of this motif are allowed. Typically, these sequences consist of a small residue, followed by a basic one and a hydrophobic residue.

The *PEX5* gene encodes the receptor, Pex5p that specifically recognizes the PTS1. *PEX5* genes have been described from various organisms including yeast, trypanosomes, plant and mammals. Mutations in the human *PEX5* gene are the cause of severe peroxisomal disorders like Zellweger syndrome and neonatal adrenoleukodystrophy [2–4].

Pex5p binds the PTS1 of newly synthesized proteins in the cytosol and subsequently guides the cargo-protein to a docking site at the peroxisomal membrane. In 2001, Dammai and Subramani [5] presented compelling evidence that human Pex5p is a cycling receptor, which upon binding to a PTS1-cargo protein, associates with the peroxisomal membrane, translocates across this membrane and finally, upon release of its cargo, recycles to the cytosol. This so-called 'extended shuttle model' is also very likely to occur in *Hansenula polymorpha*, a methylotrophic yeast that is used extensively as a model organism for studies on peroxisome biogenesis and degradation [6].

The N-terminal half of Pex5p has been shown to be important for association of the protein with the peroxisomal surface. In this region a number of conserved di-aromatic pentapeptide repeats are present that specifically bind to the cytosolic domain of the peroxisomal membrane protein Pex14p with high affinity [7,8]. The C-terminal half of Pex5p is responsible for recognition of the PTS1 tripeptide. Sequence comparisons of Pex5ps from various organisms revealed that this region contains highly conserved TPR (tetratricopeptide) repeats. The consensus sequence of this 34-amino acid repeat consists of a pattern

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Abbreviations: PTS, peroxisomal targeting signal; HpPex5p, *Hansenula polymorpha* Pex5p; HsPex5p, human Pex5p; DNS, 5-dimethylamino-naphtalene-1-sulfonyl; FRET, fluorescence resonance energy transfer.

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of small and large hydrophobic residues and has been found in a wide variety of proteins involved in diverse cellular processes (e.g. cell cycle control, transcription regulation, protein folding, protein translocation and regulation of phosphate turnover [9,10]).

Here we used fluorescence techniques to characterize *H. polymorpha* Pex5p (HpPex5p). Our data indicate that the conformation of HpPex5p is highly dependent on pH. Addition of dansylated PTS1 peptides caused some shortening of the average fluorescence lifetime of the Trp residues. These results are discussed in relation to a three-dimensional model of the C-terminal domain of HpPex5p based on the three-dimensional structure of human Pex5p [11].

Materials and methods

Organisms and growth

H. polymorpha Δpex5::URA3 leu 1.1 [6] was grown in batch cultures at 37 °C on mineral medium [12] supplemented with 0.5% (v/v) methanol as carbon source together with 0.25% (w/v) ammonium sulphate as nitrogen source. *Escherichia coli* DH5α and M15 [pREP4] were grown on Luria–Bertani medium supplemented with the appropriate antibiotics [13].

PTS1 peptides

Two dansylated PTS1 peptides, purchased from Eurosequence (Groningen, the Netherlands), were used. Both peptides contained 5-dimethylamino-naphtalene-1-sulfonyl (DNS) attached to their N termini. Peptide L1 (dansyl-C_6-ASSASKL) was coupled to DNS via a spacer of six methylene groups, whereas L2 (dansyl-GSKL) was coupled directly to DNS. The concentration of the peptides was determined spectrophotometrically using the molar extinction coefficient of $\varepsilon_{345} = 3.5 \times 10^4$ M·cm^{-1} for DNS.

Molecular techniques

Standard recombinant DNA techniques [13] and transformation of *H. polymorpha* were performed as described previously [14]. Restriction enzymes and biochemicals were from Roche (Almere, the Netherlands) and used as detailed by the manufacturer.

Construction of a C-terminal His-tagged HpPex5p

To facilitate HpPex5p purification, a C-terminal His_6-tagged protein was produced in *E. coli*. To this purpose first a *PEX5* PCR-product was obtained using primers 5′-GCG CCATGGCATTTCTGGGAGGATCGG-3′ and 5′-CGC AGATCTTATGTCGTAGGTTTTTCGG-3′. The PCR-product was cloned as a 1.7-kb *Nco*I–*Bgl*II fragment (sites introduced by the PCR-primers used) into vector pQE-60 (Qiagen) and the resulting plasmid was transformed to *E. coli* M15 [pREP4]. Transformants were grown as detailed in The QIA*expressionist*™ and induced by addition of 1 mM isopropyl thio-β-D-galactoside and incubation for 3 h at 30 °C. All subsequent steps were performed at 4 °C. Cells were harvested by centrifugation, resuspended in 50 mM phosphate buffer, pH 7.8, containing 300 mM NaCl, 1% Tween-20, 0.2 mM 2-mercaptoethanol, 10% glycerol, 0.2 mM $MgSO_4$, 10 mM imidazole, 1 mM phenylmethylsulphonyl fluoride, Complete™ (Roche), 0.1 mM EDTA, 50 μg·mL^{-1} DNAse and 100 μg·mL^{-1} RNAse and homogenized using a cell disrupter at 15 Psi. Cell debris and insoluble material were removed by centrifugation (10 000 ***g***, 20 min). The supernatant was incubated for 1 h with Ni–NTA–agarose resin (Qiagen). Subsequently, the resin was washed with 10 column vols 50 mM potassium phosphate buffer pH 7.0, containing 100 mM NaCl and 40 mM imidazole, followed by elution using 3 column vols of the same buffer containing 200 mM imidazole. HpPex5p peak fractions were loaded onto a Mono-Q HR 5/5 anion exchange column (Amersham-Pharmacia). Bound proteins were eluted using a linear gradient from 0.1 to 1 M NaCl in 20 mM Bis/Tris buffer pH 7.0. HpPex5p peak fractions were subjected to gel filtration using a Superose-12 column HR 10/30 (Amersham-Pharmacia) and 50 mM potassium phosphate buffer pH 7.2, containing 300 mM NaCl. HpPex5p-His_6 was detected by Western blotting using antibodies against HpPex5p [6] or the His_6 tag.

The concentration of HpPex5p was determined spectrophotometrically using a molar extinction coefficient of 5.8×10^4 M^{-1}·cm^{-1} at 280 nm, which was calculated on the basis of its aromatic amino acid content [15].

Expression of PEX5-HIS_6 in an *H. polymorpha* deletion strain

The functionality of the HpPex5p–His_6 fusion protein was tested by introducing the encoding gene under control of the *PEX5* promoter in an *H. polymorpha pex5* deletion strain (*Δpex5*). To this purpose the *PEX5–6HIS* cassette was isolated as a 1.8-kb *Nco*I (blunted)–*Hin*dIII fragment from the pQE60-*PEX5–6HIS* plasmid and ligated into the shuttle vector pHS5 together with a 0.5-kb *Bam*HI (blunted) *Sac*I *PEX5* promoter fragment (*Bam*HI-site introduced by PCR). The resulting plasmid was transformed to *H. polymorpha Δpex5::URA3 leu 1.1*. Transformants were tested for complementation of the methanol-growth defect of *H. polymorpha Δpex5*.

Fluorescence measurements

All measurements were performed at room temperature (22 °C) using purified HpPex5p (final concentration 0.5 μM) in 50 mM potassium phosphate buffer containing 300 mM NaCl at different pH values. PTS1 peptides were added at a concentration of 5 μM.

Steady-state fluorescence was measured using a Perkin-Elmer model MPF-43 spectrofluorometer equipped with a thermostatically controlled cuvette holder. The relative Trp emission quantum yield (Q_{Trp}) was determined by comparing the integrated fluorescence spectrum of the protein excited at 295 nm (λ_{exc} 295, emission determined at the emission maximum) with that of the standard N-Ac-Trp-NH_2 normalized to the same absorbance at 295 nm. A value of 0.13 was used for the quantum yield of the standard [16]. Quenching of Trp fluorescence (λ_{exc} 295, emission determined at emission maximum) was measured at pH 7.2

or pH 6.0. Acrylamide was used as external quencher. The data were analysed according to the Stern–Volmer equation [16]:

$$F_0/F = 1 + K_Q[X]$$

where F_0 and F are the fluorescence emission intensities in the absence and presence of acrylamide, respectively; [X] is the molar concentration of acrylamide and K_Q represents the overall quenching constant. As heterogeneous Trp emission was observed, the modified Stern–Volmer equation was applied:

$$F_0/(F_0 - F) = 1/[X]\Sigma f_a K_Q + \Sigma K_Q/\Sigma f_a K_Q$$

allowing calculation of the fraction (f_a) and the effective quenching constant (K_Q) for the most accessible class of Trp chromophores [16].

The pH dependence of the Trp fluorescence was studied in the pH range 5.6–8.5. The protein samples were incubated at 4 °C at each pH for 15 h prior to the measurements.

Time-resolved fluorescence and fluorescence anisotropy were measured using the time-correlated single photon counting technique described earlier (for example for Trp fluorescence and anisotropy decays [17]). The excitation wavelength was 300 nm and the fluorescence was measured through a Schott (Mainz, Germany) interference filter with maximum transmission at 348.8 nm. Analysis of the fluorescence intensity decay and anisotropy decay was performed with a model of discrete exponentials using the TRFA Data Processing Package of the Scientific Software Technologies Center of the Belarusian State University, Minsk, Belarus (details in [18]).

Molecular modelling of HpPex5p

To investigate the binding of the PTS1 peptide and correlate the data obtained from Trp fluorescence analysis, a molecular model of HpPex5p was built using the programs WHAT IF [19] and YASARA [20]. With 38% sequence identity to the known structure of the human homologue, solved at 2.2-Å resolution [11] (PDB entry 1FCH), model building was straightforward except for three regions: a predicted helix [21] at residues 374–386 placed at a disordered region in the X-ray structure, and two terminal helices (residues 519–532 and 550–566) which had to be manually aligned due the absence of significant sequence identity. The model covers residues 254–568; coordinates are available from the authors upon request.

Addition of the L2 peptide was possible due to the conserved binding site and the presence of a YQSKL pentapeptide in the modelling template 1FCH.

Results

Expression and purification of HpPex5p

In order to obtain purified *H. polymorpha* Pex5p (HpPex5p), a C-terminally His_6-tagged version was expressed in *E. coli* and purified using Ni–NTA affinity chromatography. Fractions enriched in HpPex5p were further purified using anion exchange chromatography and gel filtration, which resulted in the isolation of a homogeneous preparation of HpPex5p (Fig. 1).

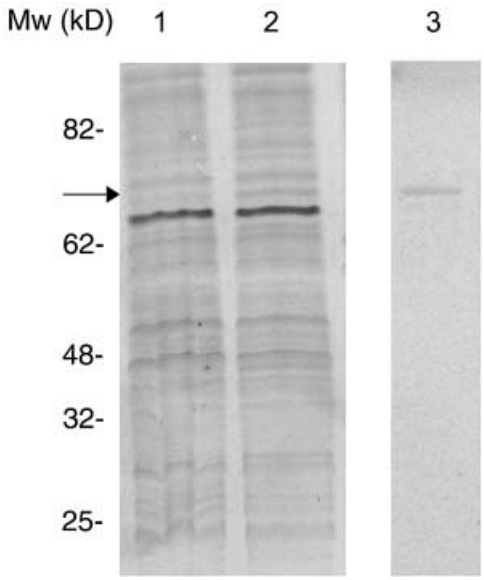

Fig. 1. Purification of HpPex5p. His_6-tagged HpPex5p was produced in *E. coli*. Lane 1, protein staining of a crude cell extract of noninduced cells; lane 2, crude extract of induced cells, containing an additional protein band at ≈ 73 kDa, the calculated molecular mass of HpPex5p. Lane 3, purified HpPex5p protein obtained upon Ni–NTA affinity chromatography, anion exchange chromatography and gel filtration.

To test whether the addition of the His_6-tag to the C-terminus of HpPex5p affected its function *in vivo*, the *H. polymorpha PEX5-HIS6* gene was expressed in an *H. polymorpha pex5* deletion strain under control of its own promoter. Growth experiments revealed that these strains grew on methanol at rates similar to wild-type cells (data not shown), indicating that PTS1 protein import was fully restored. This indicates that the His_6-tagged version of Pex5p is fully functional as PTS1 receptor.

Fluorescence properties of HpPex5p

Although HpPex5p contains twice as many Tyr as Trp residues (7 Trp and 14 Tyr residues [6]), the fluorescence emission spectra of purified HpPex5p were completely dominated by Trp emission. When excited either at 275 nm, where both Tyr and Trp absorb, or at 295 nm, where predominantly Trp absorbs, emission maxima were registered at 341 and 343 nm, characteristic of Trp chromophores located in a relatively polar microenvironment. The contribution of the Tyr chromophores to the overall protein fluorescence, as calculated from the difference emission spectra excited at 275 and 295 nm, amounted to 25–30%.

The Trp emission intensity was essentially constant in the pH range 8.5–6.5, but decreased abruptly by ≈ 25% between pH 6.5 and 6.1 (Fig. 2). Values of 0.038 and 0.026 were calculated for the Trp emission quantum yields at pH 7.2 and pH 6.0. To test whether this observed changes in Trp fluorescence were due to global conformational perturbations of the protein, acrylamide quenching of Trp fluorescence was analysed at pH 7.2 and 6.0. These experiments

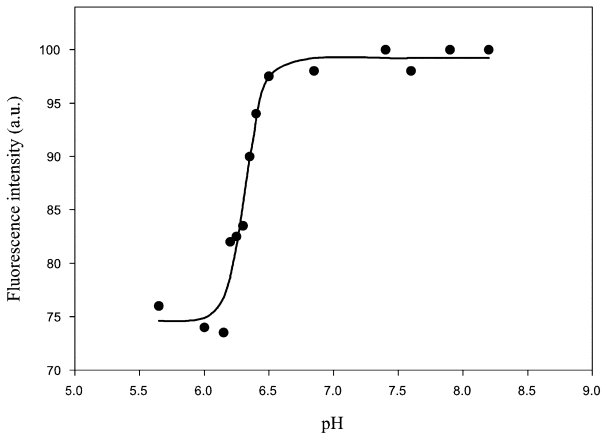

Fig. 2. pH-dependent Trp fluorescence of HpPex5p. HpPex5p was incubated for 15 h, at 4 °C, at different pH values ranging from 5.6 to 8.5. Trp emission at pH 8.0 arbitrarily was set to 100%.

revealed a downward curvature of the Stern–Volmer quenching plots at both pH values, which indicates a heterogeneous distribution of the emitting Trp chromophores [16]. Therefore, the experimental data were transformed by the modified Stern–Volmer equation allowing calculation of the fraction (f_a) and the effective quenching constant (K_Q) of the exposed Trp chromophores accessible to the quencher. The values of f_a and K_Q calculated from the data obtained at pH 7.2 were 74% and 8.6 M^{-1} and for those measured at pH 6.0 26% and 15.3 M^{-1}. As both parameters (f_a and K_Q) depend on the exposure of the Trp chromophores to the solvent, the differences in these values at the two pH values, most likely reflect a pH-related conformational change of the protein. Lowering the pH to 6.0 causes a decrease in the accessibility of the emitting Trp chromophores as suggested by the almost threefold reduction of the fraction of the exposed Trp (f_a) which, however, was more effectively quenched ($K_Q = 15.3\ M^{-1}$).

Time-resolved fluorescence

The fluorescence decay of Trp residues in HpPex5p was found to be highly heterogeneous and after analysis five lifetime components were recovered (data not shown). This is not unexpected considering the presence of seven Trp residues. In the presence of dansylated PTS1-peptides a redistribution of the lifetime patterns was observed, which led to to a shorter average lifetime (Table 1). Furthermore, these fluorescence lifetime changes were more pronounced at pH 7.2 relative to pH 6.0 showing a relatively larger reduction at pH 7.2 (Table 1). This shortening of the average fluorescence lifetime can be attributed to fluorescence resonance energy transfer (FRET) from certain Trp residues to the dansyl acceptor (for a review see [22]).

The fluorescence anisotropy decay of HpPex5p Trp residues showed different profiles depending on the pH (see Fig. 3). At both pH 7.2 and pH 6.0 they were dominated by a long decay (reflected by a large amplitude β_3), with correlation times between 39 and 63 ns at pH 6.0 (Table 1), while at pH 7.2 the decay was much slower and could not be resolved in the time range of the experiment and was fixed at 300 ns. The 39–63-ns correlation time may originate from the rotation of HpPex5p monomers and dimers (calculated molecular mass 63.9 and 127.8 kDa, respectively [6]), whereas the nonresolved correlation time may indicate the presence of larger oligomeric forms. This observation suggests the existence of different oligomeric states of the protein controlled by pH. The shorter correlation times (see Table 1) can be attributed to local Trp flexibility and resonance energy transfer among Trp residues.

Molecular modeling of HpPex5p conjugated to peptide L2

To investigate the binding of the dansylated PTS1 peptide L2 to HpPex5p and correlate the fluorescence data with structural data, a molecular model of the HpPex5p was built based on the known structure of the N-terminal domain of the human homologue, solved at 2.2 Å resolution [11]. The obtained model covers residues 254–568 of HpPex5p, corresponding to the C-terminal half of the protein including all TPR repeats (Fig. 4). Addition of the L2 peptide (dansyl-GSKL) was safely possible due to the conserved binding site and the presence of a YQSKL pentapeptide in the modelling template. Modelling of the longer peptide L1 (dansyl-C_6-ASSASKL) into a single position in HpPex5p was not possible. Because of its larger size modelling resulted in several different possibilities.

Table 1. Average fluorescence lifetimes ($\langle\tau\rangle$) and rotational correlation times (ϕ) of HpPex5p and HpPex5p complexed with L1 or L2 at pH 7.2 and pH 6.0. χ^2 varied between 1.00 and 1.05 for all experiments. ns, nanoseconds.

	pH 7.2			pH 6		
	HpPex5p	*HpPex5p + L1*	*HpPex5p + L2*	*HpPex5p*	*HpPex5p + L1*	*HpPex5p + L2*
$\langle\tau\rangle$, ns	1.78 ± 0.04 [a]	1.40 ± 0.02	1.31 ± 0.03	1.43 ± 0.08	1.31 ± 0.04	1.25 ± 0.05
β_1	0.035 ± 0.004	0.042 ± 0.008	0.031 ± 0.007	0.041 ± 0.011	0.033 ± 0.012	0.033 ± 0.012
β_2	0.089 ± 0.004	0.089 ± 0.006	0.088 ± 0.007	0.062 ± 0.008	0.058 ± 0.021	0.070 ± 0.010
β_3	0.136 ± 0.002	0.124 ± 0.001	0.137 ± 0.002	0.153 ± 0.006	0.154 ± 0.005	0.145 ± 0.006
ϕ_1, ns	0.33 ± 0.08	0.55 ± 0.13	0.42 ± 0.14	0.50 ± 0.14	0.63 ± 0.34	0.53 ± 0.21
ϕ_2, ns	2.50 ± 0.21	3.48 ± 0.80	2.32 ± 0.26	1.92 ± 0.44	2.13 ± 0.85	2.46 ± 0.62
ϕ_3, ns	300 [b]	300 [b]	300 [b]	57 ± 8	39 ± 4	63 ± 11

[a] Errors obtained from standard error analysis; [b] Value was fixed during analysis.

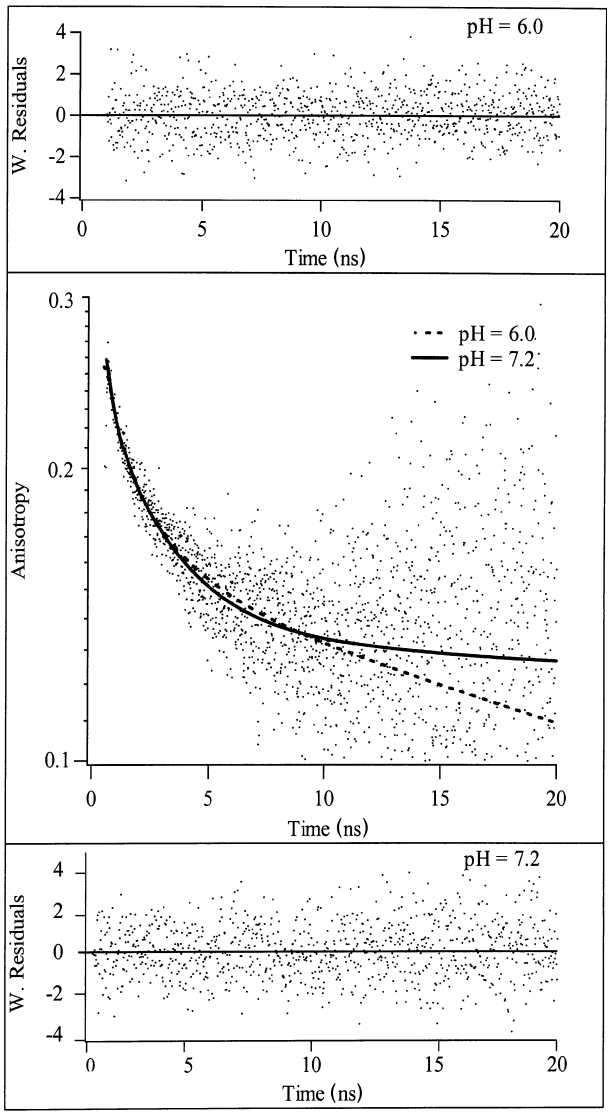

Fig. 3. Experimental (dots) and fitted (dashed and solid lines) fluorescence anisotropy decays of Trp residues in HpPex5p at two different pH values on a semilogarithmic scale (central panel). Upper and lower panels represent weighted residuals between experimental and fitted points of which the randomness around zero illustrates the goodness of fit. The anisotropy decay at pH 7.2 is distinctly slower than that at pH 6.0. The recovered parameters (amplitudes, correlation times and standard errors) are given in Table 1.

The main contacts, involving residues N352, N454, N462 and N489 that bind the PTS1 backbone as well as E320 that forms a salt bridge with lysine in L2, are entirely conserved. Only the hydrophobic pocket harbouring the terminal leucine is more pronounced in HpPex5p, including residues I348, I351 and Y427.

To analyse whether the reduction in average fluorescence lifetime could be due to FRET from certain Trp residues in the C-terminus of HpPex5p to the dansyl cap in the PTS1 peptides, the distance between the dansyl cap in L2 and the three tryptophan residues in the model were measured. The values obtained were 32 Å for W309, 28 Å for W366 and

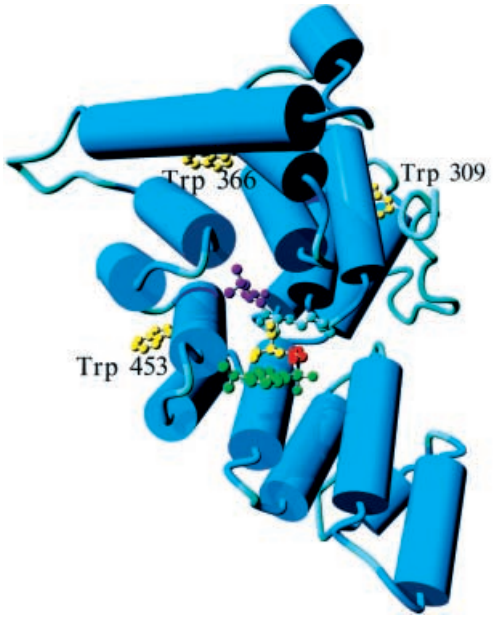

Fig. 4. Model of the C terminal domain of HpPex5p. Molecular model of the TPR domain of HpPex5p (residues 254–568) complexed with the dansylated PTS1 peptide L2. The three Trp residues (Trp309, 366 and 453) present in this domain of the protein are indicated. The L2 ligand is colour-coded: dansyl, green; Gly, red; Ser, yellow; Lys, cyan; Leu, magenta.

22 Å for W453. Because the critical distance for FRET is 21 Å [22] most likely only W453 is involved in FRET.

Discussion

In this study we investigated with fluorescence spectroscopy the conformational properties of HpPex5p, the PTS1 receptor of the yeast *H. polymorpha*. Fluorescence emission spectra revealed a pH dependence of Trp fluorescence of HpPex5p, indicative of pH driven changes in the molecule conformation. Quenching of Trp fluorescence by acrylamide showed that the fraction of accessible Trp residues (f_a) as well as the quenching constants (K_Q) associated with this fraction strongly depend on pH. Apparently, the protein is capable of adopting at least two different conformations whose distribution is controlled by pH.

The fluorescence anisotropy decay data suggested that the oligomeric state of HpPex5p differs with pH. At pH 6.0 HpPex5p was predominantly monomeric, whereas at neutral pH the protein was in an oligomeric state. These differences in oligomeric state may be responsible for the observed differences in Trp fluorescence of HpPex5p.

Previously, Schliebs *et al.* [7] demonstrated that human Pex5p (HsPex5p) forms tetramers. This conclusion was based on sizing chromatography performed at pH 8.0. However, using the same technique at a different pH (pH 7.4) Otera *et al.* [23] suggested that rat and Chinese hamster Pex5ps most likely form dimers. A possible explanation for these apparently contradictory data is that Pex5p can exist in different oligomeric states and is mainly monomeric at slightly acidic pH, but can adopt different oligomeric states at higher pH values.

It is tempting to speculate that the pH-dependent changes in HpPex5p conformation also occur *in vivo* during the extended shuttle function of HpPex5p [6] and might be a way to modulate its properties. In the cytosol (neutral pH) HpPex5p has to bind to the PTS1 of newly synthesized proteins, whereas upon import into the organelle (slightly acidic pH [24,25] the PTS1 should dissociate from Pexp5p).

The Trp fluorescence decay profiles showed a slightly faster decay upon addition of dansylated PTS1 peptides. Most likely the reduction in average fluorescence lifetime is due to FRET from certain Trp residues in HpPex5p to the dansyl cap in the PTS1 peptides. The critical transfer distance R_0 at which the transfer efficiency E is 50%, is 21 Å for a Trp–dansyl pair [22]. The experimental transfer efficiency can be obtained from:

$$E = 1 - \langle \tau_{DA} \rangle / \langle \tau_D \rangle$$

where $\langle \tau_{DA} \rangle$ and $\langle \tau_D \rangle$ are the average fluorescence lifetimes of the donor (Trp) in the presence and absence of the acceptor. When the average fluorescence lifetimes presented in Table 1 (pH 7.2) are substituted in the equation for E, E was found to be between 21% (L1) and 26% (L2). If we further assume that each of the Trp residues can be equally excited and can participate in FRET, then the majority of the Trp residues must be located at a distance larger than the critical distance $R_0 = 21$ Å. This is indeed the case as can be concluded from the structural model of the PTS1-binding C-terminal domain of HpPex5p (Fig. 4). In this domain three of the seven Trp residues of HpPex5p are found. However, of these three residues only Trp453 is positioned at a critical distance. The four Trp residues, present in the N-terminal domain of HpPex5p, are further away from the dansylated PTS1 peptides and therefore unlikely to be involved in FRET.

The three-dimensional structure of the TPR domain of human Pex5p (HsPex5p) [11] revealed that the PTS1 binding site is formed by two clusters of three TPR repeats (TPRs 1–3 and TPRs 4–6) connected by a hinge region which shows some homology to TPR repeats, but does not display its characteristic three-dimensional structure. The PTS1 peptide occupies a groove between the two TPR clusters and is bound by a set of Asn residues located in TPR3, TPR5 and TPR6. Both in HsPex5p and HpPex5p a Trp residue is located next to a conserved Asn residue in TPR5 which is involved in PTS1 binding (Trp488/Asn489 in HsPex5p, Trp453/Asn454 in HpPex5p). This suggests that this residue may be mostly concerned upon peptide conjugation. The three-dimensional model of the TPR domain of HpPex5p complexed with L2 peptide confirms this hypothesis and shows that Trp453 is the chromophore located closest to the dansyl of the bound peptide, a position appropriate for dipole–dipole coupling of the chromophores. Based on the three-dimensional model we estimated that, dependent on the oligomerization state of the receptor, the distance could range from 18.5 to 25.3 Å, sufficient to allow FRET.

Because the reduction in average lifetime was most pronounced at pH 7.2 relative to pH 6.0, HpPex5p may have a higher affinity for PTS1 peptides at neutral pH compared to pH 6.0. Possibly oligomeric HpPex5p binds PTS1 proteins in the cytosol (pH 7.2), whereas the cargo is released from HpPex5p upon dissociation of the protein into monomers in the slightly acidic peroxisomal matrix. Cytosolic binding of PTS1 proteins to oligomeric HpPex5p is fully in line with the 'preimplex' model postulated by Gould and Collins [26].

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