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In vitro import of peroxisome-targeting signal type 2 (PTS2) receptor Pex7p into peroxisomes

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ABSTRACT

Pex7p, the peroxisome-targeting signal type 2 (PTS2) receptor, transports PTS2 proteins to peroxisomes from the cytosol. We here established a cell-free Pex7p translocation system. In assays using post-nuclear supernatant fractions each from wild-type CHO-K1 and *pex7* ZPG207 cells, ^{35}S-labeled Pex7p was imported into peroxisomes. ^{35}S-Pex7p import was also evident using rat liver peroxisomes. ^{35}S-Pex7p was not imported into peroxisomal remnants from a *pex5* ZPG231 defective in PTS2 import and *pex2* Z65. When the import of ^{35}S-Pex5pL was inhibited with an excess amount of recombinant Pex5pS, ^{35}S-Pex7p import was concomitantly abrogated, suggesting that Pex5pL was a transporter for Pex7p, unlike a yeast cochaperone, Pex18p. ^{35}S-Pex7p as well as ^{35}S-Pex5p was imported in an ATP-independent manner, whilst the import of PTS1 and PTS2 cargo-proteins was ATP-dependent. Thereby, ATP-independent import of Pex7p implicated that Pex5p export requiring ATP hydrolysis is not a limiting step for its cargo recruitment to peroxisomes. PTS1 protein import was indeed insensitive to N-ethylmaleimide, whereas Pex5p export was N-ethylmaleimide-sensitive. Taken together, the cargo-protein translocation through peroxisomal membrane more likely involves another ATP-requiring step in addition to the Pex5p export. Moreover, upon concurrent import into peroxisomes, ^{35}S-Pex5pL and ^{35}S-Pex7p were detected at mutually distinct ratios in the immunoprecipitates each of the import machinery peroxins including Pex14p, Pex13p, and Pex2p, hence suggesting that Pex7p as well as Pex5p translocated from the initial docking complex to RING complex on peroxisomes.

1. Introduction

Peroxisomal matrix proteins are synthesized on free polyribosomes and posttranslationally imported into peroxisomes [1], requiring the concerted function of protein import machinery [2,3]. The majority of peroxisomal matrix proteins contain peroxisome-targeting signal (PTS)[3] type 1 at the extreme C-terminus with the tripeptide SKL motif [4-6]. Newly synthesized PTS1 proteins are recognized and transported into peroxisomes by the soluble PTS1 receptor, Pex5p [7-9]. PTS2 proteins bearing N-terminal cleavable presequence (R/K)(L/V/I)X_5(H/Q)(L/A) [10-12] are recognized and transported by the PTS2 receptor, Pex7p [13-16]. In addition to Pex7p, various peroxins are involved in the PTS2 import pathway, including the longer isoform of Pex5p (Pex5pL) in mammalian cells, redundant Pex18p and Pex21p in *Saccharomyces cerevisiae* [17], and Pex20p in *Yarrrowia lipolytica* [18], *Neurospora crassa* [19], *Pichia pastoris* [20], and *Hansenula polymorpha* [21]. It was reported that Pex18p and Pex21p promoted the formation of an import competent Pex7p-PTS2 protein complex before the docking step in *S. cerevisiae* [22]. Pex5pL, Pex18p/Pex21p, and Pex20p share the conserved sequence region responsible for binding to Pex7p and N-terminal di-aromatic pentapeptide motifs [23,24]. Furthermore, N-terminal regions of *Sc*Pex5p and *Sc*Pex18p are functionally interchangeable [25], suggesting that they are functional and structural homologues. Of note, Pex5pL independently and directly binds to Pex14p and Pex13p, the constituents of initial docking complex [23], whilst Pex18p/Pex21p interact with Pex14p and Pex13p, only in the presence of Pex7p [22]. Furthermore, in mammalian cells, not in yeast, Pex7p binds to PTS2 proteins in the absence of Pex5pL and interacts with Pex14p via Pex5pL [14,26]. Thus, it remained to be elucidated whether Pex5pL functions at the Pex7p docking step to peroxisomes or at the PTS2-cargo recognition step of Pex7p [27]. After unloading the cargoes in peroxisome matrix, Pex5p and Pex7p are exported to the cytosol

Abbreviations: ADAPS, alkyl-dihydroxyacetonephosphate synthase; AMP-PNP, adenosine 5′-(β, γ-imido)triphosphate; AOx, acyl-CoA oxidase; ARS, ATP-regenerating system; CHO, Chinese hamster ovary; DHAPAT, dihydroxyacetonephosphate acyltransferase; LDH, lactate dehydrogenase; PNS, post-nuclear supernatant; PTS1 and PTS2, peroxisome-targeting signal types 1 and 2; Pex5pS and Pex5pL, shorter and longer isoforms of Pex5p, rPex5p, recombinant Pex5p; NEM, N-ethylmaleimide

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[28,29]. Such PTS receptor shuttling requires the orchestrated function of several peroxins including Pex14p, Pex13p, Pex2p, Pex10p and Pex12p [17,30-35]. Pex14p, the initial Pex5p-docking protein, plays a central role in this process. An in vitro system to reproduce the translocation of Pex5p was recently developed [36-38]. Using such import system, molecular dynamics of Pex5p was delineated. Pex5p is imported into peroxisomes in an ATP-independent manner and exported to the cytosol in an ATP-dependent manner [36-38]. Moreover, Pex1p and Pex6p of the AAA ATPase family and their anchoring protein, Pex26p (Pex15p in yeast), are essential for Pex5p export [37,38]. Ubiquitination of Pex5p is reported to be required for the recycling of Pex5p [39-41]. Thus, contrary to the better-defined mechanism of Pex5p translocation, Pex7p import pathway remains obscure, partly due to the lack of its reliable cell-free assay system.

In this work, we attempted to establish an in vitro system for Pex7p transport. Using a successfully developed cell-free Pex7p import system, we show here that Pex7p is imported into peroxisomes in a temperature- and Pex5pL-dependent and ATP-independent manner. We also found that import of acyl-CoA oxidase (AOx), a PTS1 protein, was insensitive to N-ethylmaleimide (NEM) whereas Pex5p export was NEM-sensitive. Furthermore, interaction of Pex7p with Pex14p, Pex13p, and Pex2p was distinct from that of Pex5p with these peroxins, implying that Pex7p was translocated through the import machinery complexes in a manner different from Pex5p.

2. Materials and methods

2.1. Antibodies

We used rabbit antisera to Pex14p [35], catalase [42] and cytochrome P450 reductase [43], goat antisera to lactate dehydrogenase (LDH) (Rockland), and mouse monoclonal antibodies to Tom20 and Bip (Santa Cruz Biotech) for Western blotting. For immunoprecipitation, we used rabbit antibodies to Pex2p [44], and an epitope influenza virus hemagglutinin (HA) and mouse monoclonal anti-Flag antibody conjugated to agarose (Sigma-Aldrich).

2.2. Cell culture

CHO cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum under 5% CO_2–95% air [42].

2.3. Synthesis of radiolabeled proteins

To generate a cDNA encoding N-terminally Flag-tagged Chinese hamster Pex7p, fused to T7 promoter, PCR was performed using as a template *Flag-ClPEX7* cDNA in pUcD2Hyg [14], with a set of forward primer (CGCCAAGCTCTAATACGACTCACTATAGGGAAAGGTACCATGG-ATTACAAGGACGAC) and reverse primer (TGGTTCTTTCCGCCTCCAG). To generate a cDNA fused to T7 promoter coding for C-terminally HA_2-tagged Chinese hamster mature alkyl-dihydroxyacetonephosphate synthase (mADAPS-HA_2) lacking the presequence [45], PCR was done using as a template ADAPS-HA_2 cDNA in pcDNA 3.1 [46], with a set of forward primer (ATCGATCGATCAGTCGACCTTAATACGACTCACTATAG-GGAAAGGTACCA TGAAAGCGCGGAGAGCCGCG) and reverse primer (AGAAGGCACAGTCGAGG). cDNAs encoding ADAPS-HA_2 [46], full-length rat acyl-CoA oxidase (AOx) [5], N-terminally Flag-tagged human dihydroxyacetonephosphate acyltransferase (DHAPAT) [47] (Yagita, Y. et al., unpublished), and N-terminally His_6- and Flag-tagged Pex5pL [38], the larger isoform [48,49], were also used for in vitro translation. Flag tagging to Pex7p and His_6-Flag tagging to Pex5p did not affect the biological activity of Pex7p [14] and Pex5p (K. Okumoto and Y. Fujiki, unpublished data). These cDNAs were transcribed and translated using TNT Quick Coupled transcription/translation systems (Promega) with ^{35}S-methionine and ^{35}S-cysteine (Perkin-Elmer Japan, Tokyo, Japan) as labels [38].

2.4. Expression and purification of recombinant Pex5p

Escherichia coli XL1-blue cells were transformed with cDNAs each encoding the shorter isoform of Pex5p, Pex5pS fused to glutathione-S-transferase (GST-Pex5pS), and GST-Pex5pL [48] in pGEX6-1 and were cultured overnight at 37 °C. Each culture was diluted in 100 ml of yeast extract-tryptone medium. After being cultured at 37 °C for 90 min, cells were further grown at 37 °C for 3 h in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside (Nacalai Tesque). Harvested cells were resuspended in 10 ml of ice-cold suspension buffer containing 50 mM Tris–HCl, pH 7.4/150 mM NaCl/1 mM dithiothreitol/5% glycerol, sonicated, and centrifuged to remove cell debris. The resulting supernatant was mixed with 50 μl of glutathione-Sepharose beads (GE Healthcare Biosciences) and incubated at 4 °C for 5 h. After being washed three times with the suspension buffer, recombinant Pex5p proteins were eluted from Sepharose beads by cleaving GST off with PreScission protease (GE Healthcare Biosciences) in 50-μl suspension buffer.

2.5. In vitro import assays

Assays for in vitro import of Pex7p, Pex5p, AOx, DHAPAT, and ADAPS were performed as follows. A post-nuclear supernatant (PNS) fraction was prepared from CHO-K1 and several CHO *pex* cell mutants including *pex7* ZPG207 [14,50], *pex5* ZPG231 [51], and *pex2* Z65 [44]. CHO cells (6×10^7 each) were harvested and homogenized in 0.25 M sucrose, 5 mM Hepes-KOH, pH 7.4, and 0.1% ethanol [38]. The PNS fraction was obtained by centrifuging the homogenate twice at 700 ×g for 5 min. The import reaction was performed using ^{35}S-labeled proteins and PNS (1 mg protein) in 200 μl of import buffer, 0.25 M sucrose–5 mM Hepes-KOH, pH 7.4–0.1% ethanol–5 mM methionine–3 mM $MgCl_2$–50 mM KCl. The import assay was also done with peroxisomes isolated from rat liver (see below). Import of ^{35}S-proteins to peroxisomes was verified by its resistance to the treatment with externally added protease in the absence or presence of 1% Triton X-100, as follows. The import reaction mixture was incubated on ice for 30 min with 500 μg/ml Pronase (Sigma) [14] for Pex7p and Pex5p, or 80 μg/ml Proteinase K (Sigma) for AOx, ADAPS, DHAPAT, and Pex5p. Then, the assay mixture was centrifuged at 20,000 ×g for 30 min at 4 °C to separate organelle and cytosolic fractions and analyzed by SDS-PAGE using 10% gel. ^{35}S-FLPex7p was detected by a Fujix FLA5000 Autoimaging analyzer (Fuji Film, Tokyo, Japan). In all import assays except for those in Figs. 4, 5A and D, an ATP-regenerating system (ARS) containing 1 mM ATP, 10 mM creatine phosphate (Roche Diagnostics) and 50 μg/ml of creatine kinase (Roche Diagnostics) was added. To more readily detect the imported ^{35}S-proteins, ^{35}S-proteins were immunoprecipitated as follows. After the import reaction, protease-treated organelles were sedimented, solubilized with lysis buffer: 20 mM Hepes-KOH, pH 7.4/150 mM NaCl/1% Triton X-100/1 mM dithiothreitol/1 mM EDTA/10% glycerol/complete mini EDTA free (Roche Diagnostics), and subjected to immunoprecipitation with anti-HA antibody for ^{35}S-ADAPS-HA_2 and anti-Flag antibody-conjugated agarose for ^{35}S-FLPex7p.

2.6. In vitro Pex5p export assay

Pex5p export assay was performed essentially as described in our earlier report [38]. Briefly, PNS incubated with ^{35}S-Pex5pL in the import reaction was centrifuged at 20,000 ×g for 20 min to isolate the organelles containing ^{35}S-Pex5pL-imported peroxisomes. The organelle fraction was resuspended with the cytosolic fraction in export buffer, 0.25 M sucrose/5 mM Hepes-KOH, pH 7.4/0.1% ethanol/5 mM methionine/3 mM $MgCl_2$/50 mM KCl/4% rabbit reticulocyte lysate/1 mM ATP + ARS or 10 mM adenosine 5′-(β, γ-imido)triphosphate (AMP-PNP) (Sigma-Aldrich). After the export reaction, the assay mixture was separated into organelles and cytosolic fraction by

centrifugation at 20,000 ×g for 30 min. ^{35}S-Pex5pL was detected as above.

2.7. Subcellular fractionation of rat liver

The liver of a rat that had been injected with Triton WR-1339 [6] was homogenized in 0.25 M sucrose, 5 mM Hepes-KOH, pH 7.4, 1 mM EDTA, and 0.1% ethanol. Peroxisomes were isolated by equilibrium density gradient ultracentrifugation of a light-mitochondrial fraction in 27% OptiPrep (Axis-Shield Poc, Oslo, Norway) [52] in a Beckman VTi-50 vertical rotor. Ultracentrifugation was carried out at 230,000 ×g (average) for 3 h at 4 °C. The gradient was fractionated into 35 tubes. Fractions 3 (peroxisome) and 33 (microsomes) (200 μg each protein) were diluted with buffer containing 0.25 M sucrose, 5 mM Hepes-KOH, pH 7.4, and 0.1% ethanol, followed by centrifugation at 20,000 ×g for 30 min at 4 °C. Pellets were resuspended with 100 μl of rat liver cytosol (600 μg protein). Resulting mixtures were used for import assays of ^{35}S-FLPex7p.

2.8. Immuno-detection assay

After the reaction for import of ^{35}S-FLPex7p or simultaneous import of ^{35}S-FLPex7p and ^{35}S-Pex5pL, organelle fraction was solubilized for 30 min on ice with 1% digitonin in 5 mM Hepes-KOH, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, protease inhibitor cocktail (2 μg/ml aprotinin, 25 μg/ml antipain, and 25 μg/ml leupeptin), and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 100,000×g for 15 min. Supernatant fraction was incubated with antibodies to Pex14p, Pex13p, and Pex2p or pre-immune serum on ice for 30 min. The antigen-antibody complexes were recovered with the protein A-Sepharose beads (GE Healthcare Biosciences, Tokyo, Japan) and analyzed by SDS-PAGE and a Fujix FLA5000 Autoimaging analyzer.

2.9. Western blotting

Western blotting analysis was done using electrophoretically transferred samplers on polyvinylidene difluoride membranes (Bio-Rad) with primary antibodies and second antibody, donkey anti-rabbit, mouse, or goat immunoglobulin G antibody conjugated to horseradish peroxidase (GE Healthcare Biosciences) [48]. Antigen-antibody complexes were visualized with an ECL Western blotting detection reagent (GE Healthcare Biosciences).

3. Results

3.1. In vitro import of PTS2 protein ADAPS and Pex7p into peroxisomes

To investigate molecular mechanisms underlying the protein import to peroxisomes, we attempted to establish a cell-free system for transport of the PTS2 receptor, Pex7p, as well as PTS2 proteins, basically by extending the in vitro Pex5p import assay system [38].

First, we examined the in vitro import of PTS2 protein ADAPS, the second-step enzyme in plasmalogen synthesis [53,54]. ^{35}S-labeled and C-terminally HA_2-tagged ADAPS [46], termed ^{35}S-ADAPS-HA_2, synthesized in an in vitro transcription/translation system was incubated for 60 min at 26 °C with PNS fraction from wild-type CHO-K1 cells in the presence of 1 mM ATP and ARS. The reaction mixtures were divided into three aliquots, two of which were treated with Proteinase K in the absence and presence of 1% Triton X-100. Respective samples were separated into organelle pellet (P) and cytosolic supernatant (S) fractions (Fig. 1A). Peroxisomal markers, Pex14p and catalase, and cytosolic LDH were detectable in P and S fractions, respectively, confirming the adequate separation of organelles and the cytosol (Fig. 1A, lanes 1 and 2). In the case of PNS from *pex2* Z65, catalase was detected in S fraction, indicative of the defect in

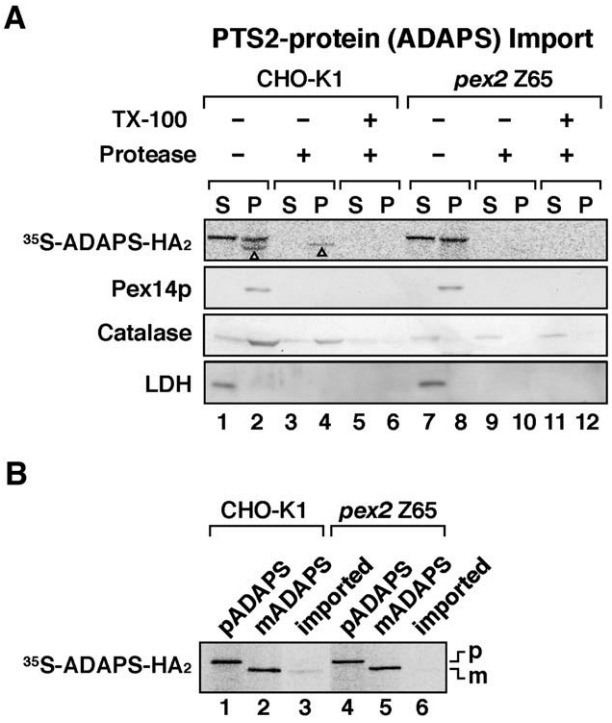

Fig. 1. In vitro import of PTS2 protein, ADAPS. (A) An in vitro import assay of PTS2-type ADAPS was performed at 26 °C for 60 min with cell-free synthesized ^{35}S-ADAPS-HA_2 and PNS fractions each from wild-type CHO-K1 (lanes 1–6) and *pex2* Z65 (lanes 7–12) cells in import buffer containing 1 mM ATP and ARS. After incubation, the reaction mixtures were mock treated (lanes 1, 2, 7 and 8) or treated with 80 μg/ml of Proteinase K for 30 min on ice (lanes 3–6 and 9–12) in the absence (−) or presence (+) of 1% Triton X-100 and were separated to organelle (P) and cytosolic (S) fractions by centrifugation. Equal aliquots of S and P fractions were analyzed by SDS-PAGE using a 10% acrylamide gel. ^{35}S-ADAPS-HA_2 was detected by a Fujix FLA-5000 autoimaging analyzer. Open arrowhead indicates the imported and processed ^{35}S-ADAPS-HA_2. Endogenous Pex14p, catalase, and LDH were detected with respective antibodies. (B) After the import reaction (A) protease-treated organelle fractions were sedimented, solubilized with 1% Triton X-100, and subjected to immunoprecipitation with anti-HA antibody. Immunoprecipitates were analyzed by SDS-PAGE (lanes 3 and 6) as in (A). In vitro translation products of the precursor ^{35}S-ADAPS-HA_2 (2.5% input used for import reaction) (lanes 1 and 4) and the mature form ^{35}S-ADAPS-HA_2 (mADAPS) (lanes 2 and 5) were also loaded.

matrix protein import [44] (lanes 7 and 8). Faster-migrating form of ^{35}S-ADAPS-HA_2 was detected in organelle pellet before and after the Proteinase K treatment (lanes 2 and 4, open arrowheads), apparently reflecting the intraperoxisomal signal processing [54]. ^{35}S-ADAPS-HA_2 of the cytosolic fractions was completely digested with the protease (Fig. 1A, lane 3), under which Pex14p exposing both N- and C-terminal parts to the cytosol and LDH, but not peroxisomal matrix enzyme, catalase, were likewise digested with the protease, hence indicating a proper protease treatment (lanes 3 and 4). Proteinase K treatment in the presence of Triton X-100 abolished the protease-resistance of the processed ^{35}S-ADAPS-HA_2 and catalase (Fig. 1A, lane 6). In contrast, with PNS fraction from *pex2* Z65 cells, the processed and protease-resistant form of ^{35}S-ADAPS-HA_2 was not detectable in the organelle fractions (lanes 8 and 10), thereby indicating the defect of PTS2 import. These results were consistent with the phenotype with respect to the PTS2 protein import of wild-type CHO-K1 cells and the peroxisome-deficient *pex* mutants, including Z65 impaired in matrix protein import [42,44]. To more readily visualize and confirm the processed- and protease-resistant form of ^{35}S-ADAPS-HA_2, we performed immunoprecipitation with anti-HA antibody using organelle fractions from the Proteinase K-treated import reaction mixtures. ^{35}S-ADAPS-HA_2 with the same migration as the mature form of ^{35}S-ADAPS-HA_2 (mADAPS) was indeed recovered from the organelle fraction of CHO-K1, not Z65 cells, (Fig. 1B), confirming that PTS2 presequence at the N-terminal part was processed, with the

HA_2-tagged C-terminus remaining intact. Taken together, ^{35}S-ADAPS-HA_2 was specifically imported into peroxisomes.

Next, we investigated peroxisomal import of Pex7p in vitro. ^{35}S-labeled and Flag-tagged Pex7p, ^{35}S-FLPex7p, was likewise incubated at 26 °C for 60 min as for ^{35}S-ADAPS-HA_2, using CHO cell-derived PNS fraction with 1 mM ATP and ARS. With PNS from wild-type CHO-K1 and *pex7* ZPG207 cells, ^{35}S-FLPex7p was detected in the organelle and cytosolic fractions (Fig. 2A, upper two panels, lanes 1 and 2). ^{35}S-FLPex7p in organelle fractions was partly resistant to the treatment with Pronase (Fig. 2A, lane 4, open arrowhead), whilst ^{35}S-FLPex7p of the cytosolic fractions was completely digested with the protease (lane 3). Taken together, these results suggested that ^{35}S-FLPex7p was imported to organelles, presumably to peroxisomes. In contrast, with the PNS fractions each from *pex2* Z65 and *pex5* ZPG231 cells, no ^{35}S-FLPex7p was detectable after the Pronase digestion (Fig. 2A, lower two panels, lanes 3 and 4), hence suggesting that ^{35}S-FLPex7p was not imported to membrane fractions, apparently peroxisome remnants so-called peroxisomal ghost. We also performed immunoprecipitation of ^{35}S-FLPex7p with anti-Flag antibody using organelle fractions from the Pronase-treated import reaction mixtures. ^{35}S-FLPex7p was indeed recovered from the organelle fractions of CHO-K1 and ZPG207, not Z65 and ZPG231, thereby confirming the Pex7p import to peroxisomes (Fig. 2B).

Next, to corroborate peroxisome-specific import of ^{35}S-FLPex7p, we performed the in vitro ^{35}S-FLPex7p import assay using subcellular fractions of rat liver. The light-mitochondrial fraction was separated by ultracentrifugation in an OptiPrep density gradient. The gradient was fractionated into 35 tubes and assayed for the distributions of marker proteins including Pex14p (peroxisomes), Tom20 (mitochondria), cytochrome P450 reductase (smooth microsomes), and Bip (rough microsomes) (Fig. 2C). Pex14p were detected with a peak at fractions 3–5, apparently free from mitochondria and microsomes

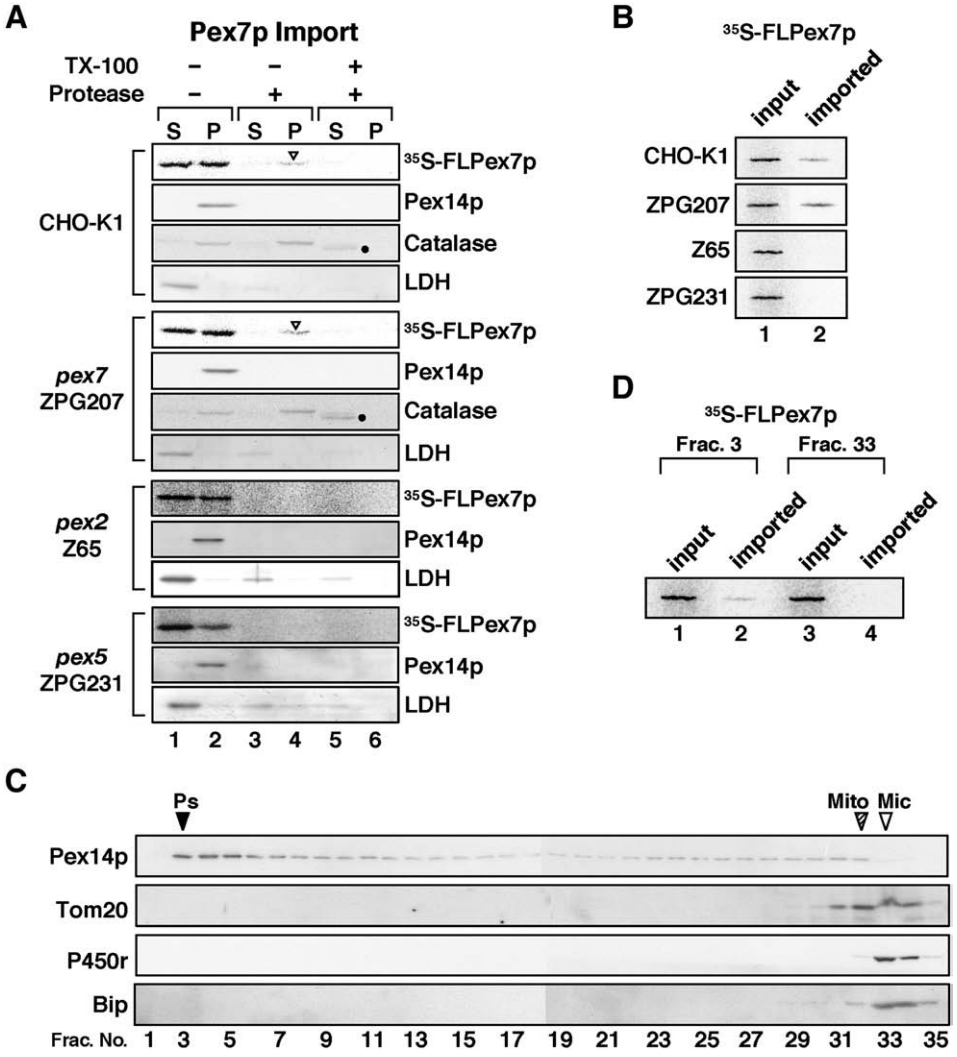

Fig. 2. In vitro import of PTS2 receptor, Pex7p. (A) An in vitro Pex7p import assay was performed at 26 °C for 60 min with cell-free synthesized ^{35}S-FLPex7p and PNS fractions each from wild-type CHO-K1, *pex7* ZPG207, *pex2* Z65, and *pex5* ZPG231 cells in the presence of 1 mM ATP and ARS. The reaction mixtures were mock treated (lanes 1 and 2) or treated with 500 μg/ml of Pronase for 30 min on ice (lanes 3–6) in the absence (−) or presence (+) of 1% Triton X-100 and were separated to organelle (P) and cytosolic (S) fractions. Equal aliquots of S and P fractions were analyzed by SDS-PAGE and ^{35}S-Pex7p was detected as in Fig. 1A. Open arrowhead indicates protease-resistant ^{35}S-FLPex7p. Endogenous Pex14p, catalase and LDH were detected as in Fig. 1A. Dot designates Pronase-cleaved fragment of catalase. (B) Imported ^{35}S-FLPex7p was immunoprecipitated from the protease-treated organelle fractions with anti-Flag antibody, as in Fig. 1B. Lanes: 1, ^{35}S-FLPex7p (2.5% input); 2, imported ^{35}S-FLPex7p. (C) Subcellular fractionation of rat liver. A light-mitochondrial fraction from rat liver was fractionated by ultracentrifugation in 27% OptiPrep. Distribution of peroxisomes, mitochondria, and microsomes was verified by Western blotting using antibodies to respective organelle marker proteins: Pex14p, Tom20, P450 reductase (P450r), and Bip. Solid, shadowed, and open arrowheads indicate the peak fractions of peroxisomes (fraction no. 3), mitochondria (fraction no. 32), and microsomes (fraction no. 33), respectively. (D) ^{35}S-FLPex7p was specifically imported into peroxisomes. ^{35}S-FLPex7p was incubated with organelles (200 μg each) from fractions no. 3 (lane 2) and no. 33 (lane 4), each supplemented with rat liver cytosol (600 μg protein) and 1 mM ATP plus ARS. ^{35}S-FLPex7p import was verified as in (B).

that were highly enriched in fractions 31–34 and 33–34, respectively. The ^{35}S-FLPex7p import assay was done separately using fractions 3 and 33, followed by immunoprecipitation as in Fig. 2B. Protease-resistant ^{35}S-FLPex7p was detected in the immunoprecipitates from the import assay mixture using the fraction 3, but not in those from the fraction 33 (Fig. 2D, lanes 2 and 4), thereby demonstrating that ^{35}S-FLPex7p was imported specifically to peroxisomes. Collectively, we concluded that an in vitro import assay system for Pex7p as well as ADAPS was successfully established with the high import specificity and physiological relevance.

3.2. Pex7p is imported to peroxisomes in a manner dependent on temperature and Pex5pL

Next, we examined temperature dependence of ^{35}S-FLPex7p import using PNS fraction from *pex7* ZPG207. In contrast to ^{35}S-FLPex7p import assay on ice where no ^{35}S-Pex7p protected from the digestion with Pronase was detected (Fig. 3A, lanes 1–6), the assay at 26 °C gave rise to protease-resistant ^{35}S-Pex7p in P fraction (lanes 7–12). The ^{35}S-Pex7p import at 26 °C was likewise confirmed by immunoprecipitation as in Fig. 2B (Fig. 3B), hence strongly suggesting that the ^{35}S-FLPex7p was imported in a temperature-dependent manner.

Next, to address whether the in vitro Pex7p import into peroxisomes is dependent on Pex5pL import, we performed the import assays of ^{35}S-FLPex7p and ^{35}S-Pex5pL in the absence and presence of rPex5pS, using *pex7* ZPG207-derived PNS fraction. In the absence of rPex5pS, ^{35}S-Pex5pL and ^{35}S-FLPex7p in the P fraction were partly protected from Protease-digestion (Fig. 3C, lanes 1–4, solid and open arrowheads), indicating that both of ^{35}S-Pex5pL and ^{35}S-FLPex7p were imported into peroxisomes. In the presence of a lager amount (10 μg) of rPex5pS, the import of ^{35}S-Pex5pL as well as ^{35}S-FLPex7p was significantly affected (Fig. 3C, lane 8). In contrast, in the presence of 10 μg of rPex5pL, ^{35}S-FLPex7p was imported (lane 12, open arrowhead), whilst the import of ^{35}S-Pex5pL was abrogated, apparently owing to being replaced with relatively abundant rPex5pL in the import reaction mixture. Immunoprecipitation of ^{35}S-FLPex7p with anti-Flag antibody confirmed and more evidently demonstrated that the imported ^{35}S-FLPex7p in the presence of rPex5pS was strikingly reduced as compared to that in the absence of rPex5pS or in the presence of rPex5pL (Fig. 3D). Taken together, these results strongly suggest that Pex7p is imported in a manner dependent on the concomitant import of Pex5pL. Distinct from yeast Pex18p/Pex21p that function at the PTS2 protein recognition step [22], Pex5pL likely plays a pivotal role at the docking step of Pex7p to peroxisomes, with preferential binding to Pex7p-PTS2-cargo complex, not free Pex7p, in the cytosol [14].

3.3. Pex7p is imported to peroxisomes in an ATP-independent manner

To verify whether the Pex7p import requires ATP, we also performed the Pex7p import assays in the presence of 1 mM ATP and ARS or 10 mM AMP-PNP, an unhydrolyzable ATP analogue. Under these two distinct conditions, nearly the same amount of ^{35}S-FLPex7p in P fractions was protected from the treatment with Pronase (Fig. 4A, lanes 4 and 10, open arrowheads; Fig. 4B, lanes 2 and 4), strongly suggesting that ^{35}S-FLPex7p was imported in an ATP-independent manner, presumably together with endogenous Pex5pL.

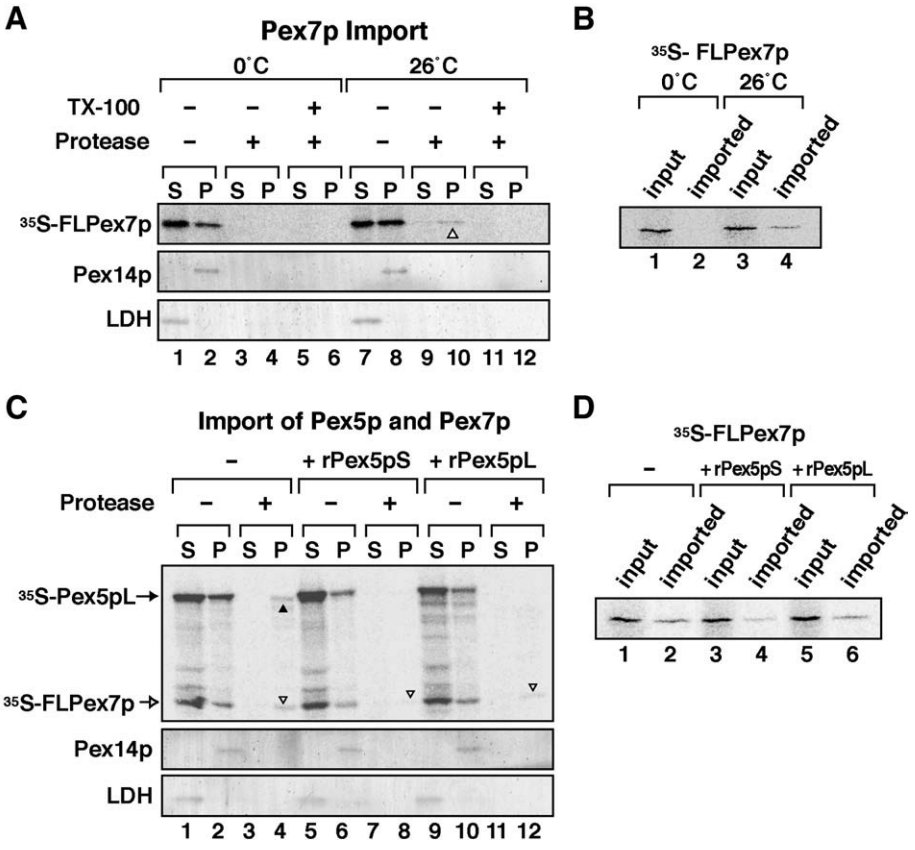

Fig. 3. Temperature- and Pex5pL-dependent import of Pex7p. (A) ^{35}S-FLPex7p was incubated with PNS from *pex7* ZPG207 for 60 min at 0 °C (lanes 1–6) or 26 °C (lanes 7–12) in the presence of 1 mM ATP and ARS. ^{35}S-FLPex7p import was assessed as in Fig. 2A. Open arrowhead indicates protease-resistant ^{35}S-FLPex7p. (B) ^{35}S-FLPex7p import was assessed by immunoprecipitation as in Fig. 2B. (C) ^{35}S-Pex5pL and ^{35}S-FLPex7p were incubated with ZPG207-derived PNS in the absence (lanes 1–4) or presence of 10 μg of purified recombinant Pex5pS (rPex5pS, lanes 5–8) or rPex5pL (lanes 9–12). Import of ^{35}S-Pex5pL and ^{35}S-FLPex7p was verified as in Fig. 2A. Solid and open arrowheads indicate protease-resistant ^{35}S-Pex5pL and ^{35}S-FLPex7p, respectively. (D) ^{35}S-FLPex7p import was assessed by immunoprecipitation as in Fig. 2B.

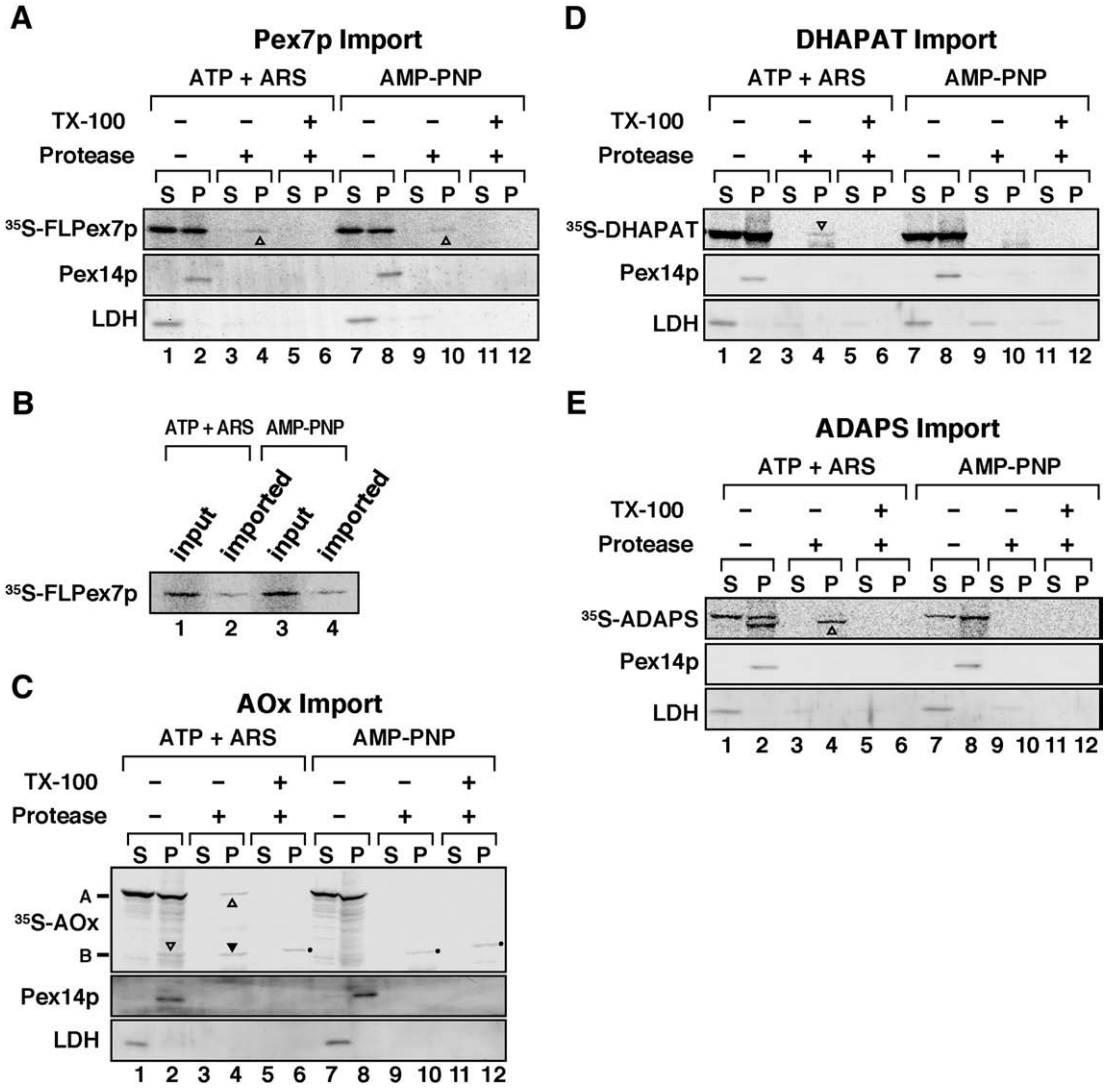

Fig. 4. Pex7p import does not require ATP hydrolysis. (A) An in vitro ^{35}S-Pex7p-import assay was likewise performed in the presence of 1 mM ATP and ARS (lanes 1–6) or 10 mM AMP-PNP (lanes 7–12). ^{35}S-FLPex7p import was verified as in Fig. 2A. Open arrowhead indicates ^{35}S-FLPex7p protected from protease digestion. (B) ^{35}S-FLPex7p import was verified by immunoprecipitation as in Fig. 2B. (C) ^{35}S-AOx import assay was likewise performed using ZPG207-derived PNS as in (A). Upward and downward open arrowheads indicate ^{35}S-AOx-A and -B chains, respectively; solid arrowhead shows apparent mixture of ^{35}S-AOx-B and a Proteinase K-cleaved fragment of ^{35}S-AOx [6]. Dots designate Proteinase K-cleaved fragment of ^{35}S-AOx. (D) ^{35}S-DHAPAT import assay was performed using CHO-K1-derived PNS as in (A). Open arrowhead indicates ^{35}S-DHAPAT. (E) ^{35}S-ADAPS import was performed using PNS from CHO-K1 as in (A). Open arrowhead indicates a processed and imported form of ^{35}S-ADAPS.

Furthermore, we likewise carried out cell-free import assay of ^{35}S-AOx with PNS from ZPG207 cells under the same conditions as in Fig. 4A. In the presence of 1 mM ATP and ARS, ^{35}S-AOx in P fraction was partly protected from the Proteinase K treatment (Fig. 4C, lane 4), indicating that ^{35}S-AOx was imported into peroxisomes. After the import reaction in the presence of 10 mM AMP-PNP, ^{35}S-AOx protected from the protease digestion was barely detectable (Fig. 4C, lane 10), consistent with the report by Imanaka et al. [55], whilst ^{35}S-AOx in P fraction was at the level comparable to that in the presence of 1 mM ATP and ARS (compare lanes 2 and 8). Hence, these results indicated that ^{35}S-FLPex7p import was not affected even under the condition which ^{35}S-AOx import was strongly interfered. Taken together, we concluded that ATP hydrolysis was not required for Pex7p import into peroxisomes.

To verify whether the ATP-dependent import is prevalent to peroxisomal matrix proteins, we performed cell-free import assays of another PTS1 protein, DHAPAT, the first-step enzyme in plasmalogen synthesis [47,56] and PTS2 protein ADAPS. ^{35}S-DHAPAT and ^{35}S-ADAPS were likewise incubated with PNS from CHO-K1 cells in the presence of 1 mM ATP and ARS (Fig. 4D and E, lanes 1–6) or 10 mM AMP-PNP (Fig. 4D and E, lanes 7–12). In the presence of 1 mM ATP and ARS, full-length ^{35}S-DHAPAT and PTS2-cleaved ^{35}S-ADAPS were protected from protease treatment (Fig. 4D and E, lane 4, open arrowheads), hence indicating that these ^{35}S-labeled proteins were imported into peroxisomes. In the presence of 10 mM AMP-PNP, ^{35}S-DHAPAT and ^{35}S-ADAPS likewise recovered in P fractions were not protected from the protease digestion (Fig. 4D and E, lane 10). No processed ^{35}S-ADAPS band was discernible either (Fig. 4E, lanes 8 and 10), thereby indicating that the import of ^{35}S-DHAPAT and ^{35}S-ADAPS was abrogated. Together, these results strongly suggested that both of the PTS1 and PTS2 proteins were imported in a manner dependent on ATP hydrolysis, consistent with earlier reports [55,57,58].

3.4. Pex5p export is not a rate-limiting step for AOx import

ATP-independent ^{35}S-FLPex7p import was unexpected, because both ^{35}S-FLPex7p import and ATP-dependent PTS1 protein transport were mediated by Pex5pL (Figs. 3 and 4). ^{35}S-Pex5pL is exported from peroxisomes in an ATP-dependent manner [36-38]. Together, the export and recycling of Pex5p may not be prerequisite for the

transport of ^{35}S-FLPex7p to peroxisomes at least in vitro, rather only one-cycle import of Pex5p may be sufficient for it.

To investigate whether or not Pex5p export is the limiting step for import of ^{35}S-AOx and ^{35}S-FLPex7p, we first performed the Pex5p export assay in the presence of AMP-PNP and NEM. Upon incubation at 26 °C for 30 min in the presence of ATP and ARS, ^{35}S-Pex5pL increased in its level in cytosolic (S) fraction with concomitant decrease of ^{35}S-Pex5pL in organelle (P) fraction, thereby indicating that ^{35}S-Pex5pL was exported to the cytosol (Fig. 5A, lanes 1–4). In contrast, in the presence of 10 mM AMP-PNP or 1 mM NEM, ^{35}S-Pex5p export was strongly abrogated (lanes 5–12), indicating that ^{35}S-Pex5p was exported in an ATP-dependent and NEM-sensitive manner. A NEM-sensitive factor(s) is likely involved in the ATP-dependent Pex5p export. In the ^{35}S-Pex5p import reaction in the presence of NEM, protease-resistant ^{35}S-Pex5p was more distinct than that in the absence of NEM (Fig. 5B, lanes 4 and 10), thereby possibly reflecting the normal import and abrogated export of ^{35}S-Pex5p. Hence, we concluded that Pex5p import was not affected with NEM.

Next, we performed ^{35}S-AOx import assays using CHO-K1-derived PNS in the presence or absence of 1 mM NEM. Under the both conditions, Proteinase K-resistant ^{35}S-AOx was detected in respective P fraction (Fig. 5C, lanes 4 and 10), strongly suggesting that ^{35}S-AOx was imported into peroxisomes. Proteinase K-resistant ^{35}S-AOx-A was detectable at a higher level in the presence of 1 mM NEM than a control (lanes 4 and 10, open arrowheads), presumably owing to the inhibition of proteolytic conversion of AOx-A to B and C (lanes 2 and 8,

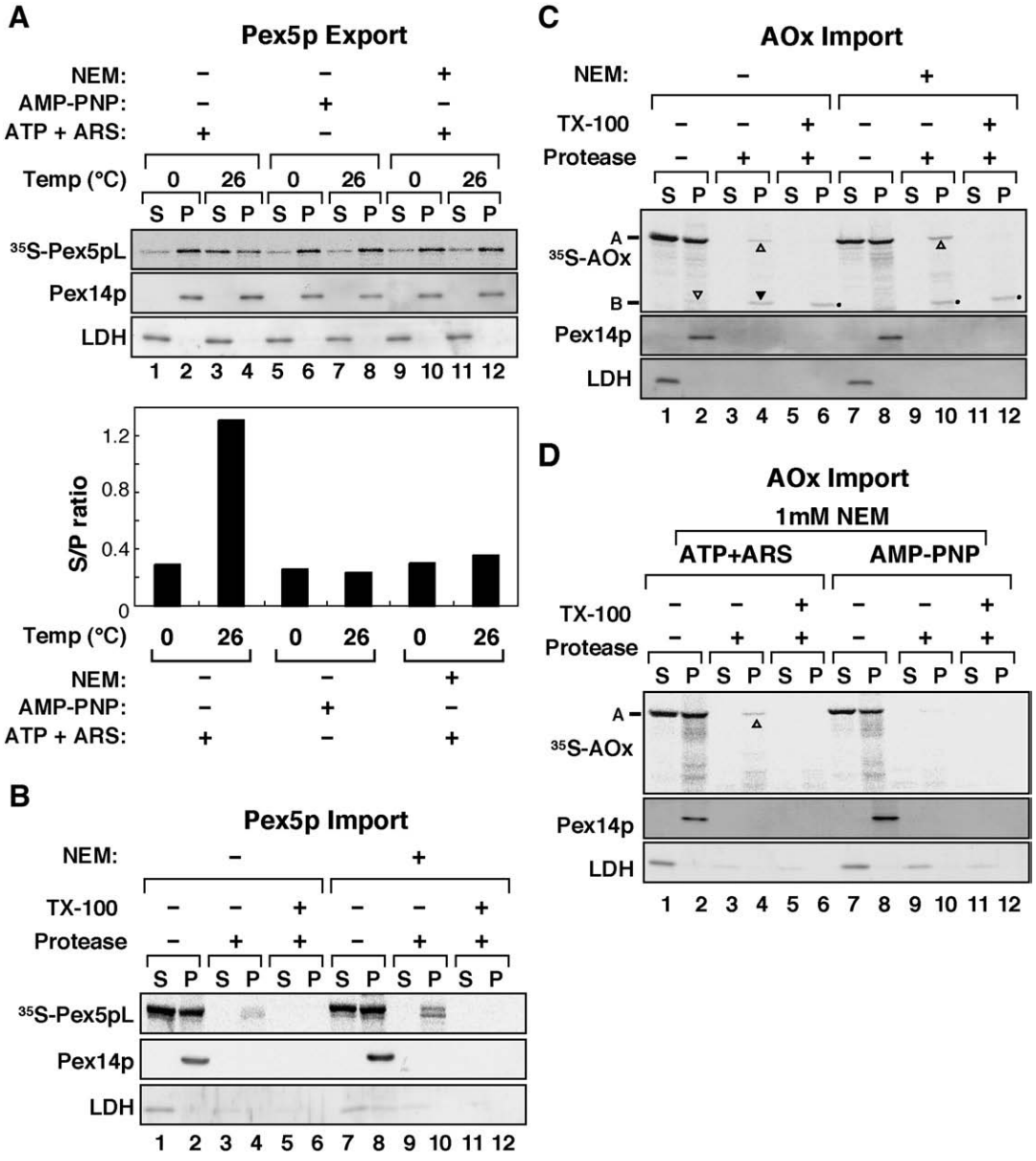

Fig. 5. Effects of N-ethylmaleimide on Pex5p export and AOx import. (A) Pex5p is exported in an ATP hydrolysis dependent and NEM-sensitive manner. ^{35}S-Pex5pL import was done with PNS from CHO-K1. Organelle fraction was resuspended with cytosolic fraction in export buffer containing 1 mM ATP and ARS (lanes 1–4 and 9–12) or 10 mM AMP-PNP (lanes 5–8). The mixtures were mock treated (lanes 1–8) or pretreated with 1 mM NEM (lanes 9–12) for 10 min on ice. Export reaction was performed for 30 min on ice (lanes 1, 2, 5, 6, 9 and 10) or at 26 °C (lanes 3, 4, 7, 8, 11 and 12). The reaction mixtures were separated to organelle (P) and cytosol (S) fractions. ^{35}S-Pex5pL in S and P fractions was detected as in Fig. 2A. (B) Pex5p import is NEM-insensitive. ^{35}S-Pex5pL import assay was performed in the absence (lanes 1–6) or presence (lanes 7–12) of 1 mM NEM. Import of ^{35}S-Pex5pL was verified as in Fig. 1A. (C) AOx import is NEM-insensitive. ^{35}S-AOx was incubated with PNS from CHO-K1 in the absence (lanes 1–6) or presence (lames 7–12) of 1 mM NEM. Import of ^{35}S-AOx was verified as in Fig. 1A. Upward and downward open arrowheads indicate AOx-A and -B chains, respectively. Open and solid arrowheads and dots are as in Fig. 4C. (D) AOx is imported in a manner dependent of ATP hydrolysis in the presence of NEM. ^{35}S-AOx was incubated with CHO-K1-derived PNS treated with1 mM NEM in the presence of 1 mM ATP and ARS (lanes 1–6) or 10 mM AMP-PNP (lanes 7–12). Open arrowhead indicates AOx-A chain.

open arrowhead). Recently, trypsin-domain-containing 1 (Tysnd1) harboring PTS1 was identified as a NEM-sensitive conversion enzyme for AOx in mammalian peroxisomal matrix [59]. The severely affected the conversion to B- and C-components of ^{35}S-AOx-A in the presence of NEM is compatible with such properties of Tysnd1. Taken together, the import of ^{35}S-AOx and ^{35}S-Pex5p is NEM-insensitive, whilst ^{35}S-Pex5p export is NEM-sensitive. Accordingly, these findings imply that Pex5p export is not essential for the import of Pex7p as well as AOx at least in vitro. Next, we tested ATP-dependent import of ^{35}S-AOx in the presence of NEM. In the presence of both 1 mM ATP plus ARS and 1 mM NEM, protease-resistant ^{35}S-AOx was detected in P fraction (Fig. 5D, lane 4 open arrowhead). On the contrary, in the presence of both 10 mM AMP-PNP and 1 mM NEM, ^{35}S-AOx was barely protected from protease digestion (lane 10). This suggested that ^{35}S-AOx import remained in a manner dependent on ATP hydrolysis even under the condition where Pex5p export was abolished. An additional step requiring ATP hydrolysis is more likely involved in the AOx import pathway.

3.5. Differential interaction of Pex5p and Pex7p with import machinery complexes

Pex5p translocates through the matrix protein import machinery on peroxisomes, comprising Pex2p, Pex10p, Pex12p, Pex13p, and Pex14p, via the initial interaction with Pex14p [48,60]. The export of Pex5p to the cytosol is catalyzed by AAA ATPase peroxins, Pex1p and Pex6p [37,38]. However, the fate of Pex7p after the translocation to peroxisomes remained largely unknown. Therefore, we searched for interacting partners, if any, of the imported ^{35}S-FLPex7p by co-immunoprecipitation analysis. After the ^{35}S-FLPex7p import reaction using CHO-K1-derived PNS fraction, the organelle fraction was solubilized with 1% digitonin and subjected to immunoprecipitation with antibodies each to Pex14p, Pex13p, and Pex2p. As shown in Fig. 6A, ^{35}S-FLPex7p was specifically co-immunoprecipitated with respective antibodies, strongly suggesting that Pex7p bound to these three peroxins on peroxisomes like Pex5p. With regard to other potential constituents such as Pex10p and Pex12p, our antibodies to Pex10p and Pex12p were not potent enough to immunoprecipitate these peroxins (data not shown). Next, to compare and characterize the Pex5p- and Pex7p-containing complexes on peroxisome membrane, we likewise immunoprecipitated Pex14p, Pex13p, and Pex2p from the import reaction mixtures containing both ^{35}S-Pex5p and ^{35}S-FLPex7p. ^{35}S-Pex5p and ^{35}S-FLPex7p were specifically co-immunoprecipitated with Pex14p, Pex13p, and Pex2p (Fig. 6B, lanes 2–5). ^{35}S-Pex5p was detected at a highest level in the immunoprecipitate of Pex14p, less in that of Pex2p, and at a lowest level in that of Pex13p (lanes 2–4, solid arrowhead). Conversely, ^{35}S-FLPex7p was distinctly detectable in the immunoprecipitate of Pex13p (Fig. 6B, lanes 2–4, open arrowhead). We interpreted these results to mean that Pex5p and Pex7p interacted with these three peroxins in a mutually differentiated manner.

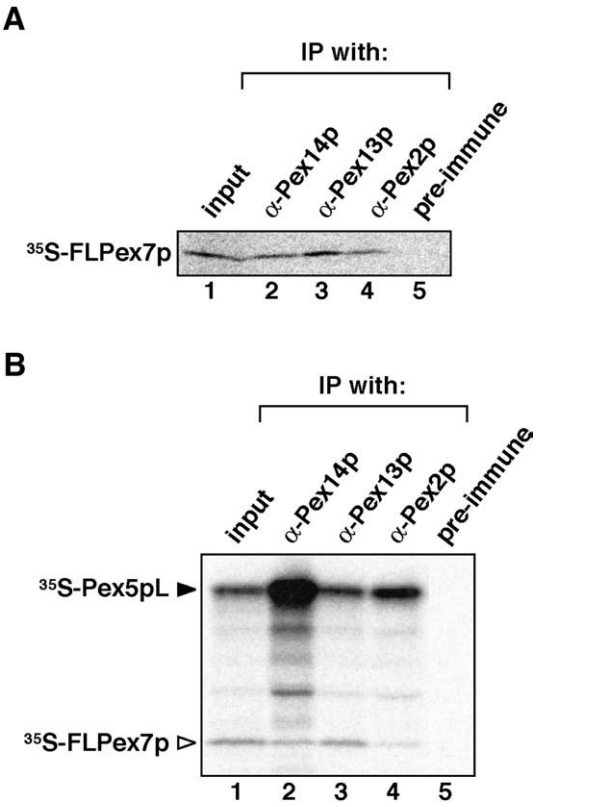

Fig. 6. Distinct complexes of Pex5p and Pex7p formed on peroxisomal membrane. In vitro import reactions were performed as in Fig. 2A, using ^{35}S-FLPex7p (A) or ^{35}S-Pex5pL and ^{35}S-FLPex7p (B) with PNS from CHO-K1 cells. Organelle fractions were sedimented, solubilized with 1% digitonin, and subjected to immunoprecipitation with antibodies against Pex14p (lane 2), Pex13p (lane 3), and Pex2p (lane 4), and with preimmune serum (lane 5). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Input (5% used for the assays) was loaded in lane 1. Solid and open arrowheads in (B) indicate ^{35}S-Pex5pL and ^{35}S-FLPex7p, respectively.

4. Discussion

In the present study, we attempted to extend our in vitro Pex5p import assay system established earlier [38] to Pex7p import. With PNS fractions from wild-type CHO-K1 and several *pex* CHO mutants including *pex7* ZPG207, we succeeded for the first time in establishing an in vitro Pex7p import assay system, which was competent for the PTS2 protein import as well. Together with in vitro assay systems for peroxisomal protein translocation that we and others earlier established [6,26,36,38,55,61], several in vitro protein import/export assay systems are now available, including the import system for PTS1 proteins, PTS2 proteins and their receptors, Pex5p and Pex7p, and the export system for Pex5p. Using this system, we investigated molecular mechanisms underlying Pex7p import into peroxisomes. ^{35}S-FLPex7p was specifically imported into peroxisomes isolated from rat liver as well as those in PNS fraction from CHO-K1 cells (Fig. 2). ^{35}S-FLPex7p was also imported into peroxisome ghosts of *pex7* ZPG207 cells (Fig. 2A), implying that peroxisome ghosts of this mutant were accommodated with the components, except for Pex7p, required for the import of Pex7p and PTS2 proteins, in compatible with the report of the restoration of protein import by microinjection of recombinant Pex5p into CHO *pex5* ZP102 cells [62]. Thus, ZPG207 is a useful tool to define the molecular basis for translocation of Pex7p in a cell-free system devoid of endogenous Pex7p. ^{35}S-FLPex7p was not imported into peroxisome ghosts of *pex2* Z65 and PTS2-import defective *pex5* ZPG231 (Fig. 2A), consistent with the *in vivo* observations [14], thereby emphasizing the specificity and physiological relevance of this in vitro assay system.

We also showed that Pex7p was imported in a manner dependent on the Pex5pL import. Hence, Pex5pL likely regulates the PTS2 import in a way different from the yeast auxiliary Pex18p/Pex21p that is involved in the PTS2 recognition of Pex7p [22]. It is more likely that Pex7p-PTS2 protein complexes as well as PTS1 proteins are intrinsic transport cargoes of Pex5pL. Moreover, ATP hydrolysis is not required for the import of Pex7p and Pex5p (Fig. 4A), suggesting that there may be similar mechanisms underlying the import of these PTS receptors. As inferred from the Pex5p export [36-38], it is plausible that Pex7p export may also require ATP hydrolysis. Due to a lower efficiency of the export of Pex7p in a cell-free system (data not shown), however, we have been so far unable to establish such an in vitro assay system.

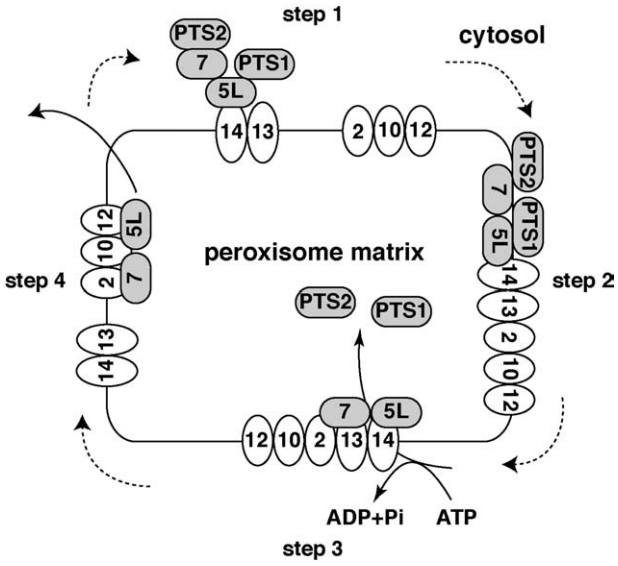

Fig. 7. A schematic model for protein translocation in matrix protein import through peroxisomal membrane. Translocation of matrix proteins and their receptors is divided to four steps. Step 1: complexes of PTS proteins–PTS receptors represented by PTS1-Pex5pL-Pex7p-PTS2 target peroxisomes via Pex5p-mediated interaction with the initial docking protein, Pex14p. Step 2: PTS receptors, Pex5p and Pex7p, translocate across the peroxisomal membrane in an ATP-independent manner, whilst PTS1 and PTS2 proteins remain on the cytosolic phase of peroxisomes. Step 3: PTS proteins translocate across the peroxisomal membrane by an unidentified mechanism. This step requires ATP hydrolysis energy. Step 4: PTS receptors transit from the docking complex to RING complex and exit in an ATP-dependent manner. The numbers indicate corresponding peroxins.

We found that Pex5p export, not Pex5p import, was highly NEM-sensitive. NEM-sensitive factors responsible for the Pex5p export may well be the AAA peroxins, Pex1p and Pex6p, as reported [37,38]. Very recently, a highly conserved cysteine residue at the N-terminus of Pex5p was reported to be a ubiquitination site and required for the Pex5p recycling [40,63-65]. This cysteine of Pex5p is plausible to be a target of NEM during the Pex5p export reaction. A potent inhibitor of Pex5p export, NEM, would be a useful agent for stabilizing and characterizing an intermediate complex involved in Pex5p export steps.

^{35}S-AOx import was insensitive to NEM in vitro whereas Pex5p export was highly NEM-sensitive, apparently suggesting that Pex5p export was not a limiting step for ^{35}S-AOx import. However, Imanaka et al. [66] reported that ^{35}S-AOx import was nearly 90% inhibited by NEM treatment. The difference between these two independent findings may be due to the different materials used in the assays. We used PNS fraction containing the cytosol for in vitro AOx import assay, whilst they used highly purified peroxisomes and omitted the cytosol fraction. It is more likely that our assay system contains enough cytosolic pool of Pex5p and only one-cycle import of Pex5p is sufficient to transport ^{35}S-FLPex7p and ^{35}S-AOx to peroxisomal surface. Nevertheless, ATP hydrolysis is prerequisite for ^{35}S-AOx import, consistent with the earlier report [55], suggesting that AOx, not PTS receptors, requires ATP hydrolysis to translocate through the peroxisomal membrane. In our cell-free assays, another PTS1-type protein, DHAPAT, and PTS2-type protein ADAPS were imported as well in an ATP-dependent manner (Fig. 4), hence evidently suggesting that ATP-dependent import is common to peroxisomal matrix proteins.

The observation of the difference in ATP requirement between the import of PTS1 and PTS2 proteins and that of PTS receptors lead us to propose potentially distinct machineries each catalyzing the translocation of matrix proteins and the import of their receptors across the peroxisomal membrane (Fig. 7). In *P. pastoris pex20* mutant defective in Pex20p, an auxiliary peroxin of Pex7p, 3-ketoacyl-CoA thiolase, a PTS2 protein, remained on peroxisomal surface, despite the fact that Pex7p was imported into peroxisomes [20]. This notion is similar to our findings in the present in vitro study with regard to the differential import of PTS1 and PTS2 proteins and PTS receptors, supporting our working model. It is equally possible that Pex5p and Pex7p imported prior to the matrix proteins form "transient pore" [67], then directly mediating the translocation of matrix proteins across the membrane. The physiological consequence and detailed mechanisms of such differential translocation of matrix proteins and their receptors are currently unknown. We should await further investigations to delineate such mechanisms at the molecular level.

As to the translocation of Pex7p, we demonstrated the interactions of Pex7p to the constituents of potential matrix protein import machinery including Pex14p, Pex13p and Pex2p. The first demonstration that Pex7p interacts with RING peroxin Pex2p on peroxisomes suggests that Pex7p transits the RING complex after targeting peroxisomes via the initial docking complex comprising Pex14p and Pex13p (Fig. 7), as Pex5p does [38,68]. ^{35}S-FLPex7p is not imported into peroxisome ghosts of *pex2* Z65, implying that Pex2p possibly functions at a translocation step of Pex7p through peroxisomal membrane. Pex7p also interacts with Pex13p on peroxisomes (Fig. 6), consistent with the report that Pex13p plays a pivotal role in PTS2 import pathway [26,69]. The interaction between Pex7p and Pex13p is not likely via Pex5pL, rather direct [26]. More cell biochemical approaches such as that using crosslinkers may shed light to the issues in regard to PTS2 protein import machinery. We also noted that ^{35}S-FLPex7p did not interact with the AAA peroxins essential for the export of PTS1 receptor, Pex5p [37,38] in this cell-free system (data not shown). This may be one of reasons for unsuccessful reconstitution of the Pex7p export reaction. Whether or not the AAA peroxins are involved at this step remains to be defined.

In either event, the cell-free assay system for Pex7p import reported here is a highly useful method to elucidate the mechanisms underlying PTS2 import pathway.

Acknowledgments

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