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Protein Transport into Higher Plant Peroxisomes[1]

In Vitro Import Assay Provides Evidence for Receptor Involvement

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Peroxisome biogenesis requires that proteins be transported from their site of synthesis in the cytoplasm to their final location in the peroxisome matrix or membrane. Glyoxysomes are a class of peroxisomes found primarily in germinating seedlings and are involved in mobilizing fatty acids via the glyoxylate cycle and the β-oxidation pathway. We have used an in vitro assay to study the mechanism(s) of import of proteins into glyoxysomes. Results from this assay indicate that the transport process is time- and temperature-dependent and is specific for peroxisomal proteins. Isocitrate lyase, a glyoxysomal protein, and the leaf-type peroxisomal enzyme glycolate oxidase (GLO) were transported into pumpkin (*Cucurbita pepo*) glyoxysomes with no apparent differences in efficiency of import. Thus, this in vitro assay appears to be physiologically relevant and correlates well with expected in vivo conditions. Protein import was also energy-dependent and saturable. Nonradiolabeled GLO competed with radiolabeled, in vitro-synthesized GLO for components of the import machinery. Finally, pretreatment of the isolated glyoxysomes with protease virtually abolished subsequent import of GLO. Taken together, these results indicate that a proteinaceous receptor is involved in the import of peroxisomal proteins.

Glyoxysomes are a class of higher plant peroxisomes containing enzymes unique to reactions of the glyoxylate cycle and β-oxidation of fatty acids (Olsen and Harada, 1995; Gietl, 1996). Cotyledons of most plants contain glyoxysomes that are involved in lipid mobilization to provide nutrients during germination and seedling growth. Leaves, roots, and mature pollen each possess a distinct class of peroxisomes, each performing physiological functions specific to the tissue (Olsen and Harada, 1995; Gietl, 1996). However, all peroxisomes contain catalase and other hydrogen peroxide-metabolizing enzymes and are bound by a single membrane. The interconversion of peroxisome function appears to be transcriptionally regulated, i.e. the organelle takes on the metabolic role defined by the enzymes coded by the mRNA expressed at a given time in a particular tissue (Comai et al., 1989b; Ettinger and Harada, 1990; Zhang et al., 1993). Thus, the import of proteins into peroxisomes is not developmentally regulated; glyoxysomes are competent to import leaf-peroxisomal proteins and leaf peroxisomes can import glyoxysomal proteins (Olsen et al., 1993; Onyeocha et al., 1993).

There are several signals that target proteins to peroxisomes (reviewed in Olsen and Harada, 1995; Gietl, 1996). The first peroxisomal targeting signal identified, PTS1, is a consensus tripeptide of Ser-Lys-Leu (some conserved substitutions are allowed) at the extreme carboxyl terminus of the protein. IL from *Brassica napus*, localized exclusively in the matrix of glyoxysomes in developing seeds and seedlings, possesses a PTS1 that is both necessary and sufficient for targeting to higher plant peroxisomes (Olsen et al., 1993). Many other peroxisomal proteins from diverse organisms, including plants, have been shown to share identical or similar carboxyl-terminal amino acid residues that function as peroxisomal targeting determinants. The second type of peroxisomal targeting signal, PTS2, found on a minority of peroxisomal proteins, is a short amino-terminal prepiece that is proteolytically cleaved in plants and mammals, but not in yeast. In contrast, a few peroxisomal proteins, including acyl-CoA oxidase from *Candida tropicalis*, appear to possess internal targeting signals that do not resemble PTS1 or PTS2 (Small et al., 1988; Kamiryo et al., 1989; Gietl, 1996).

Peroxisomal proteins with different PTSs may follow different import pathways, utilizing separate PTS1 and PTS2 receptors as well as some common components (reviewed in Rachubinski and Subramani, 1995; McNew and Goodman, 1996). Yeast and human cells that are selectively deficient in the import of PTS1 proteins, PTS2 proteins, or both have been identified. Recently, proteins that may function as receptors for the transport of PTS1 proteins have been identified in yeast and in humans. Similarly, candidate proteins for the PTS2 receptor have been reported in yeast. However, the subcellular localization of these proteins is somewhat controversial and varies depending on the source of the receptor protein (Rachubinski and Subramani, 1995; McNew and Goodman, 1996). The PTS1 receptor has been reported to be tightly associated

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Abbreviations: GLO, glycolate oxidase; IL, isocitrate lyase; PTS1, peroxisomal targeting signal type 1; PTS2, peroxisomal targeting signal type 2.

with the peroxisomal membrane of *Pichia pastoris*, to be largely cytosolic in humans, and to partition between the cytosol and the peroxisome matrix in *Hansenula polymorpha*. The probable PTS2 receptor from *Saccharomyces cerevisiae* may shuttle between the cytosol and the peroxisome or it may be present in the matrix. Homologs of these receptors have not yet been identified from plant peroxisomes.

The existence of at least two import pathways utilizing receptors with conflicting subcellular localization has led to the development of rather complex models of peroxisomal protein import (Rachubinski and Subramani, 1995; McNew and Goodman, 1996). Adding to the complexity of the proposed models are recent studies suggesting that folded proteins and oligomers are imported intact into peroxisomes (reviewed in McNew and Goodman, 1996).

The mechanism for import of proteins into peroxisomes appears to be common to all organisms (Olsen and Harada, 1995; Gietl, 1996); however, not all organisms and tissues possess enough peroxisomes to be feasible systems with which to study the protein translocation process. The cotyledons of oil-seed plants such as pumpkin (*Cucurbita pepo*), cotton, and watermelon are an especially abundant source of glyoxysomes that can be relatively easily isolated and used for import studies (Mori and Nishimura, 1989; Behari and Baker, 1993; Olsen and Harada, 1995; Gietl, 1996). We have taken advantage of this to develop an in vitro procedure for studying the import of proteins into isolated glyoxysomes from pumpkin cotyledons. Peroxisomal protein import is clearly a unique and complex process that now requires cell biological and biochemical approaches in addition to standard genetic analyses to answer questions about its molecular mechanisms and to thoroughly understand the cellular requirements and components associated with the translocation machinery.

In general, in vitro assays for protein transport complement and significantly expand the information accessible with in vivo experiments (Behari and Baker, 1993; Purdue and Lazarow, 1994; Lazarow, 1995; Olsen and Harada, 1995; Gietl, 1996). Additionally, a precise investigation of the molecular mechanisms involved in protein import requires the ability to study the process in vitro. Although others have developed similar assays (Mori and Nishimura, 1989; Behari and Baker, 1993), we have begun with a rigorous characterization of the physiological parameters of protein import. This is necessary to firmly establish the reliability and relevance of the in vitro assay for the study of peroxisomal protein import. Using this assay we found that transport was time-, temperature-, and energy-dependent, as well as saturable and specific for peroxisomal proteins, indicating that the assay is physiologically relevant. As a first step toward addressing more mechanistic questions, we have also used this in vitro assay to investigate receptor involvement in the process of protein import into higher plant peroxisomes.

MATERIALS AND METHODS

Preparation of Radiolabeled Peroxisomal Proteins

Plasmids containing full-length cDNA inserts for IL and malate synthase from *Brassica napus* were described previously (Comai et al., 1989a, 1989b). The plasmid containing the full-length cDNA for spinach GLO was provided by Dr. Chris Somerville (Volokita and Somerville, 1987). Dr. Elizabeth Vierling kindly provided cDNA clones for the cytosolic heat-shock protein HSP18 (DeRocher et al., 1991). IL, malate synthase, and GLO cDNA inserts were each subcloned into the pGEM7Zf(+) expression vector (Promega). The resulting plasmids were linearized with an appropriate restriction enzyme. Transcription of the linearized DNA with SP6 RNA polymerase was as described previously (Olsen et al., 1989). HSP18 was transcribed according to the directions supplied with the T7 transcription kit from Novagene (Madison, WI). Radiolabeled polypeptides (incorporating ^{35}S-Met [Amersham]) were synthesized in a cell-free wheat germ lysate system (Olsen et al., 1989).

Isolation of Pumpkin Glyoxysomes

Pumpkin seeds (*Cucurbita pepo* var. Happy Jack, Half Moon, or Big Moon, supplied by Petoseed Co., Inc., Saticoy, CA) were imbibed in running water for at least 4 h and then grown in damp vermiculite for 4 to 6 d at 25 to 28°C in complete darkness. Approximately 40 g of cotyledons were harvested manually in dim light and homogenized in grinding buffer (20 mM pyrophosphate, 1 mM EDTA, 0.3 M mannitol, and 1 mg mL^{-1} BSA, pH 7.5) using a blender (Waring) with four 3- to 5-s bursts. The crude homogenate was filtered through two layers of Miracloth (Calbiochem). Glyoxysomes were isolated essentially as described previously (Mori and Nishimura, 1989; Kato et al., 1995). The filtrate was centrifuged in a swinging bucket rotor (HB-6 rotor, Sorvall) at 4°C for 10 min at 3,000*g*. The lipid layer was carefully skimmed from the surface of the supernatant, which was then decanted into fresh tubes and centrifuged for 20 min at 10,500*g* (swinging bucket rotor; 4°C). Using a small paintbrush, the pellets were resuspended with modified isolation buffer (10 mM Hepes/KOH, pH 7.2, 1 mM EDTA, 0.3 M mannitol, and 0.1 μg mL^{-1} each antipain, leupeptin, and 100 μg mL^{-1} pepstatin), pooled, and carefully loaded onto a 28% Percoll (in modified isolation buffer) cushion overlaid on a 2 M Suc pad. The gradient was centrifuged in a swinging bucket rotor at 4°C for 30 min at 18,000*g* (without brake). Partially purified glyoxysomes were collected from a visible band at the interface between the Percoll gradient and the Suc cushion. The glyoxysomes were washed once with modified isolation buffer (swinging bucket rotor, 4°C, 14 min, 7,000*g*) prior to final resuspension. The purity of the isolated organelles was assessed by electron microscopy and by marker enzyme assays. Small amounts of contaminating mitochondria were occasionally detected (data not shown).

Leaf peroxisomes were isolated similarly, except that the pumpkin seedlings were grown in the dark for 2 d and then in the light for 4 more d to allow full "greening" of the cotyledons.

Import Reactions and Analysis

Standard import reactions (modified from those described in Behari and Baker, 1993) contained 10 μL of

glyoxysomes (approximately 400–500 μg of protein), 1 to 10 μL of translation products, and 2 to 5 mM ATP (with 2–5 mM $Mg[OAc]_2$ or as Mg^{+2} salt) in import buffer (25 mM Mes-KOH, pH 6.0, 0.5 M Suc, 10 mM KCl, 1 mM $MgCl_2$; sometimes also with 10 mM NaN_3) in a final volume of 50 to 200 μL. Other additions to the assays are indicated in the figure legends or text. Unless otherwise noted, import reactions were initiated by the addition of translation products and incubated at 22 to 26°C for 20 to 30 min. Following import, reactions were treated with proteinase K to digest translation products that were not imported. Protease treatments were performed for 30 min on ice; reactions were stopped by the addition of PMSF (1 mM final concentration) to inhibit proteinase K. To repurify intact glyoxysomes containing protease-protected imported proteins, each import reaction was layered onto a 0.7 M Suc cushion (500 μL in import buffer) and centrifuged at 8500*g* for 15 min in a refrigerated microcentrifuge. The pellets were resuspended in SDS-PAGE sample buffer, heated at 90°C for 2 to 5 min, and stored at −20°C until further analysis. Some samples (indicated in the figures) were sonicated or treated with Triton X-100 to lyse the organelles. Because sonication can cause heating of the samples, four cycles of 30-s sonication followed by 30 s on ice were used to achieve a total of 2 min of sonication. Radioactive proteins were visualized by fluorography and autoradiography.

Quantitation of the import reactions was achieved by incubating the manually excised radioactive gel bands in 30% hydrogen peroxide overnight at 50°C. A standard scintillation cocktail for aqueous samples was then added and the samples were counted on a liquid scintillation counter (model LS 6800, Beckman) to determine the relative levels of radioactive protein present in each lane of the gel.

For the competition experiments, glyoxysomes in the import cocktail were preincubated with the unlabeled proteins for 15 min at 22 to 26°C before radiolabeled GLO was added. All subsequent treatments were as described above. Nonradiolabeled GLO ($[NH_4]_2SO4$ solution, Sigma) was precipitated with TCA (15% final concentration), neutralized with NaOH, and resuspended in import buffer prior to incubation with glyoxysomes.

To proteolytically digest proteins exposed on the surface of the glyoxysomal membrane, isolated glyoxysomes were first incubated with trypsin and chymotrypsin (0.1 mg mL^{-1} each, final concentration) for 20 min at room temperature. The protease digestion was stopped by adding trypsin-chymotrypsin inhibitor (1 mg mL^{-1} final concentration). In separate experiments, isolated glyoxysomes were pretreated with either proteinase K (10 μg mL^{-1}) or thermolysin (0.1 mg mL^{-1}) on ice for 30 min. These digestion reactions were inhibited by 1 mM PMSF or 25 mM EDTA, respectively. Subsequent treatment of the import reactions was as described above. Posttreatment of the import reactions with proteinase K was used to remove any remaining bound (but not translocated) translation products. Glyoxysomes that were pretreated with proteinase K were subjected to thermolysin treatment following import because PMSF may still have been present from the pretreatment.

RESULTS

Import of the Glyoxysomal Protein IL into Isolated Pumpkin Glyoxysomes

Glyoxysomes were isolated from pumpkin cotyledons and radiolabeled IL was prepared as described in "Materials and Methods." Import reactions containing purified glyoxysomes, radiolabeled IL translation products, and ATP were incubated at room temperature (25°C) for 30 min. Unlike precursor proteins targeted to chloroplasts and mitochondria, IL is synthesized at its mature size and therefore is not proteolytically processed to a lower-molecular-weight species after import. Thus, to distinguish between imported and unimported forms of the protein in in vitro assays, we assumed that IL free in the medium and/or bound to the surface of the glyoxysomes would be digested by exogenous protease, whereas imported IL would be protease-resistant. Therefore, following import, glyoxysomes were treated with the protease proteinase K. To confirm that protease-resistant IL was imported into the organelle, sonication in the presence of protease was used to lyse the glyoxysomes and release imported IL for subsequent protease digestion. These controls form the basis of the in vitro protein import assay.

As shown in Figure 1, full-length IL was imported into glyoxysomes under these conditions. Sonication of the import sample in the absence of protease had no effect on the amount of IL associated with glyoxysomes (Fig. 1, compare lanes 1 and 2). In the absence of sonication, approximately 31% of the radiolabeled IL remained protease-resistant (Fig. 1, lane 3); this roughly represented the amount of IL that was translocated across the membrane. Protease present during sonication was sufficient to digest all imported IL that was made accessible by lysis of the or-

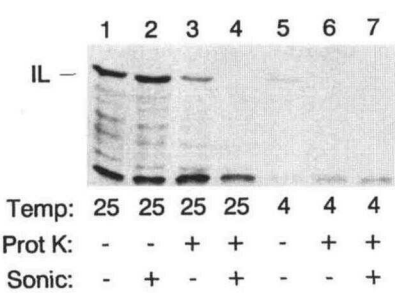

Figure 1. In vitro import of IL into isolated pumpkin glyoxysomes. Isolated pumpkin glyoxysomes were incubated with radiolabeled IL for 30 min at 25°C or at 4°C. After the import reaction, intact glyoxysomes were reisolated through a 10% Percoll cushion. The glyoxysome pellet was resuspended in import buffer (lanes 1, 2, and 5) or 10 μg mL^{-1} proteinase K (lanes 3, 4, 6, and 7) and incubated on ice for 30 min. Some samples (lanes 2, 4, and 7) were sonicated during this period to lyse the organelles. PMSF was added to all tubes to stop the protease reactions. Samples were prepared for analysis by SDS-PAGE and fluorography as described in "Materials and Methods." An autoradiograph after a 6-h exposure is shown. No IL was detected in lane 6, even after a much longer exposure of the gel.

ganelles (Fig. 1, lane 4). Protease treatment of translation products in the absence of organelles also completely digested radiolabeled IL, indicating that the protease-resistant IL in the in vitro import assays was not due simply to aggregation of the protein into a protease-protected form (data not shown). Because it was essential that this assay be consistent and reproducible, these positive and negative controls were repeated many times with independent preparations of glyoxysomes and batches of radiolabeled peroxisomal proteins, and uniformly consistent results, such as those shown in Figures 1 and 3, were obtained.

A consistent pattern of lower-molecular-weight species was seen following the import of IL (and GLO, see below). These proteins were likely due to a combination of internal initiations during translation (yielding shorter, import-competent proteins containing the carboxyl-terminal targeting signal) and degradation of the full-length protein during the import reaction, possibly due to endogenous proteases. Similar lower-molecular-weight products were observed by Baker and colleagues while studying the import of IL and GLO into sunflower glyoxysomes (Behari and Baker, 1993; Horng et al., 1995).

Physiological Characterization of Peroxisomal Protein Import

To accurately reflect the in vivo process, we expected the in vitro assay to meet certain physiological criteria. First we tested the effect of low temperature on the transport of proteins into glyoxysomes. Protein import into other organelles is known to be temperature-dependent (Eilers et al., 1988; Keegstra et al., 1989; Lazarow et al., 1991). When standard import reactions were incubated at 4°C, a small amount of IL was associated with the glyoxysomes, but it was completely protease-sensitive (Fig. 1, lanes 5 and 6), indicating that the transport process was temperature-dependent.

In addition, although IL is localized only in glyoxysomes in vivo, IL is also found in leaf peroxisomes when expressed constitutively in transgenic plants, suggesting that leaf peroxisomes are competent to import IL in vivo (Olsen et al., 1993; Onyeocha et al., 1993). Therefore, we expected that IL could be imported into leaf peroxisomes in vitro as well. Pumpkin seedlings were grown in the light to allow the cotyledons to expand and become dark green. Leaf peroxisomes were isolated from this tissue using the procedure described in "Materials and Methods" for glyoxysome preparation. Sufficient quantities of organelles were obtained for the control import samples necessary to show that radiolabeled IL was imported into leaf peroxisomes and became protease-resistant (Fig. 2).

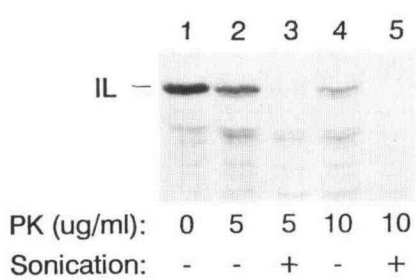

Figure 2. In vitro import of IL into isolated leaf peroxisomes from pumpkin seedlings. Standard in vitro transport assays were performed as described in "Materials and Methods," except that IL was imported into isolated leaf peroxisomes. Radiolabeled IL was incubated with isolated leaf peroxisomes and 1 mM ATP for 20 min at 26°C. After import, proteinase K was added to the samples as indicated below the gel lanes. Sonication (lanes 3 and 5) was used to lyse the organelles. All subsequent sample treatments were as described in Figure 1. A typical autoradiograph is shown.

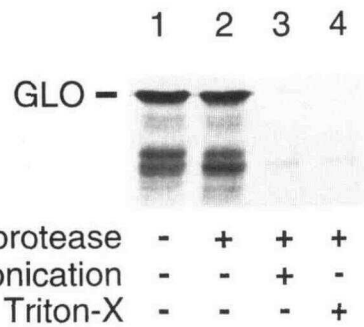

Figure 3. In vitro import of GLO into isolated pumpkin glyoxysomes. Standard in vitro transport assays were performed as described in "Materials and Methods." Radiolabeled GLO was incubated with isolated glyoxysomes and 1 mM ATP for 20 min at 26°C. After import, 10 $\mu g\ mL^{-1}$ proteinase K was added to the samples in lanes 2 through 4. Sonication (lane 3) or Triton X-100 (lane 4; 1% final concentration) was used to lyse the organelles. All subsequent sample treatments were as described in Figure 1. A typical autoradiograph is shown.

The specificity of this in vitro import system was tested to confirm that other peroxisomal proteins were imported under the same conditions first used with IL, and that extraperoxisomal proteins were not transported into the isolated organelles. First we assayed the import of malate synthase, an enzyme from the glyoxylate cycle in glyoxysomes that is coordinately expressed with IL (Comai et al., 1989b). Full-length malate synthase, radiolabeled by in vitro translation, was imported into isolated glyoxysomes under standard import conditions (data not shown). Next, we tested whether the isolated glyoxysomes were able to import the leaf peroxisomal protein GLO. The in vitro import of radiolabeled GLO into isolated pumpkin glyoxysomes is shown in Figure 3. In the experiment shown, approximately 84% of the GLO present was protease-resistant, i.e. imported into the glyoxysomes (Fig. 3, lane 2). Protease-protected GLO was digested when the organelles were lysed by sonication or by treatment with the detergent Triton X-100 (Fig. 3, lanes 3 and 4). The import of GLO into isolated glyoxysomes was also temperature-dependent; no protease-resistant GLO was seen when the reactions were incubated for 30 min at 4°C (Fig. 4A, compare lanes 3 and 4).

To determine whether the in vitro import assay was specific for peroxisomal proteins, we challenged isolated glyoxysomes with the small cytosolic HSP18 protein and

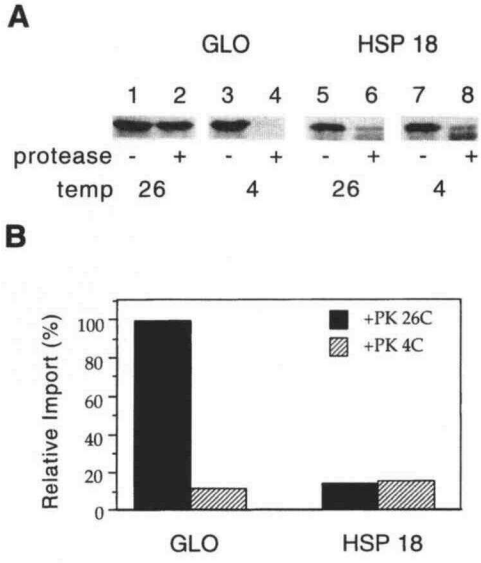

Figure 4. Import of GLO into glyoxysomes is temperature-dependent and specific for peroxisomal proteins. A, Isolated glyoxysomes were incubated with radiolabeled GLO (lanes 1–4) or the cytosolic protein HSP18 (lanes 5–8) under standard import conditions (see "Materials and Methods") at either 26°C (lanes 1, 2, 5, and 6) or 4°C (lanes 3, 4, 7, and 8). The samples in even-numbered lanes were treated with 10 μg mL^{-1} proteinase K. B, Quantitation of the protease-resistant (imported) proteins present after incubation of the import reactions at 26°C or 4°C, corresponding to lanes 2, 4, 6, and 8 in A. The amount of cytosolic HSP18 that is protease-resistant was not affected by temperature, indicating that it is not truly imported into the organelle. GLO is imported at 26°C, but not at 4°C.

with a chloroplast precursor protein. At 26°C virtually all of the radiolabeled HSP18 protein was protease-sensitive (Fig. 4B). However, a small amount of protein appeared to be protease-resistant, suggesting that some HSP18 may have been imported into the glyoxysomes (Fig. 4A, lane 6). When the import reaction was performed at 4°C, approximately the same amount of HSP18 remained after protease treatment (Fig. 4B). Because import does not occur at 4°C (Fig. 1, lane 3 versus lane 6; Fig. 4B), we concluded that the protease-protected HSP18 did not represent protein that had truly been imported into the organelle. Similarly, the 23-kD subunit of the oxygen-evolving complex of PSII was not imported into glyoxysomes (data not shown).

Next we examined the effect of varying the concentration of ATP present in the import reactions. Because the radiolabeled proteins were synthesized in the presence of ATP and an ATP-regenerating system, the translation products were desalted on a Sephadex G-25 column to remove ATP (and other small molecules) prior to the import reactions (Olsen et al., 1989). In addition, 10 mM NaN_3 was included in the import buffer during the energetics experiments to inhibit any ATP synthesis by the small amounts of mitochondria that may have been present in the glyoxysome preparation (see "Materials and Methods"). As expected (Lazarow et al., 1991; Horng et al., 1995), the level of GLO imported into glyoxysomes increased with the addition of ATP (Fig. 5).

The time-dependence of in vitro protein import into glyoxysomes is presented in Figure 6. Standard import reactions (containing 5 mM ATP) were incubated at 26°C from 0 to 60 min. The amount of GLO imported increased over time, with a peak at about 30 to 40 min; after that it appeared to level off or decrease slightly.

Involvement of a Proteinaceous Receptor in Peroxisomal Protein Import

Next we examined three criteria that should be met if the process of protein transport across peroxisomal membranes is receptor-mediated: (a) import should be saturable, (b) import should be competitive in nature, and (c) protease treatment of the glyoxysomes should inhibit protein import by compromising the function of the surface receptors. As shown in Figure 7, when increasing amounts of radiolabeled GLO were added to standard import reactions (26°C, 30 min, 5 mM ATP), import into glyoxysomes became saturated at relatively low levels of GLO. Similarly, when decreasing amounts of glyoxysomes were incubated with a constant level of radiolabeled GLO, saturation of the process was seen (data not shown).

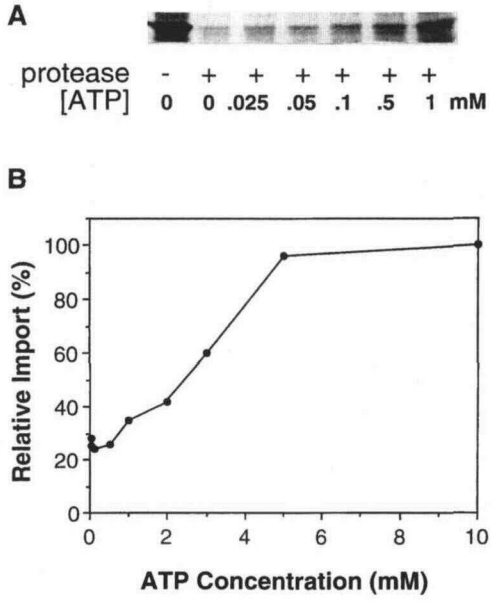

Figure 5. Import of GLO into glyoxysomes is ATP-dependent. To determine whether energy is required during the import process, increasing concentrations of ATP were added to standard import reactions. Prior to addition to the import reactions, GLO translation products were desalted on a Sephadex G-25 column to remove nucleotides remaining from the in vitro translation reaction. A, Representative autoradiograph of a concentration curve from 0 to 1 mM ATP; 10 μg mL^{-1} proteinase K was added to the samples indicated after import. B, Quantitation of import samples from a typical ATP concentration curve (0–10 mM), as described in "Materials and Methods." All of these samples were treated with 10 μg mL^{-1} proteinase K added following import to remove bound and loosely associated proteins. The amount of radiolabeled GLO imported in the presence of 10 mM ATP was set as 100% for comparison with the other samples from the same experiment.

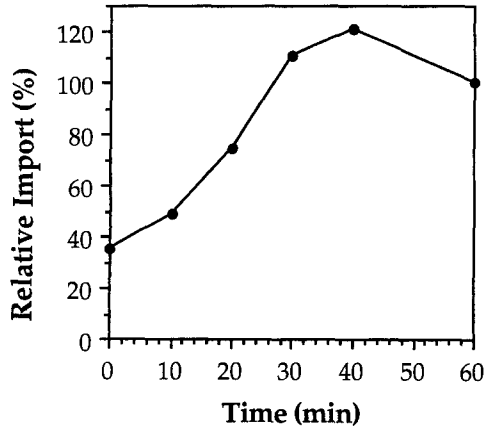

Figure 6. Import of GLO into glyoxysomes is time-dependent. Standard in vitro import reactions containing isolated pumpkin glyoxysomes, GLO translation products, and 5 mM ATP were performed for 3 to 60 min at 26°C, as described in "Materials and Methods." At the indicated times, samples were placed on ice to stop the import reaction and treated with 10 μg mL^{-1} proteinase K. The results from a typical experiment are shown. The amount of GLO imported after 60 min was set at 100% for comparison with the other time points from this experiment.

In addition, an excess of unlabeled peroxisomal proteins should saturate all putative receptor sites and block the import of radiolabeled proteins into glyoxysomes. Preincubation of glyoxysomes with excess levels of nonradioactive GLO prior to the addition of the radiolabeled protein decreased the amount of protease-resistant (imported) radiolabeled GLO observed (Fig. 8). When BSA was used as a nonspecific protein control, it did not affect the import of

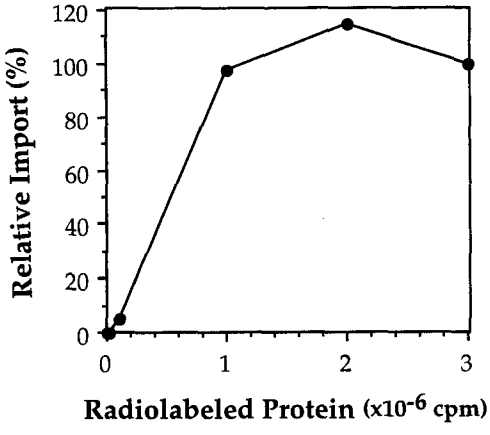

Figure 7. Import of GLO into pumpkin glyoxysomes is saturable. Isolated glyoxysomes were incubated with increasing amounts of radiolabeled GLO in standard import reactions with 5 mM ATP for 20 min at 26°C. The samples were treated with 10 μg mL^{-1} proteinase K after import; subsequent analysis and quantitation were as described in "Materials and Methods." The radiolabeled GLO translation products were added to the import samples as TCA-precipitable counts ranging from 10^3 to 3×10^6 cpm. The X-axis indicates the amount of GLO added ($\times 10^{-6}$ cpm). For comparison with other samples in this experiment, 100% relative import was set as the amount of GLO imported in the sample in which 10^6 cpm TCA-precipitable protein was added to initiate import.

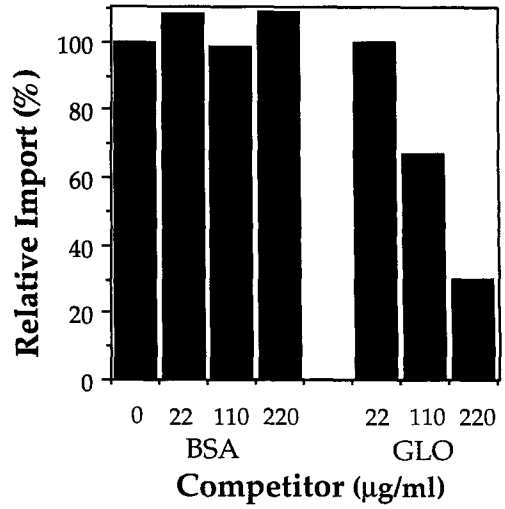

Figure 8. Nonradiolabeled GLO competes with radiolabeled GLO in vitro for transport into pumpkin cotyledons. Pumpkin glyoxysomes were preincubated with excess nonradiolabeled GLO or BSA (as a control for nonspecific protein inhibition) for 15 min at 26°C prior to the addition of radiolabeled GLO. All subsequent manipulations of the samples were as described in "Materials and Methods"; all samples were treated with 10 μg mL^{-1} proteinase K. The amount of radiolabeled GLO imported in the absence of competitor was set as 100% relative import.

GLO, suggesting that BSA did not compete with radiolabeled GLO for receptor sites on glyoxysomes (Fig. 8).

If proteinaceous receptors exposed to the cytoplasm are involved in peroxisomal protein import, then protease digestion should abolish import into the organelle. To test this hypothesis, isolated glyoxysomes were pretreated with a combination of trypsin and chymotrypsin for 20 min at room temperature. The amount of GLO imported into the glyoxysomes was greatly reduced after the protease pretreatment (Fig. 9, compare lanes 2 and 4), which did not reduce the amount of protein bound to the glyoxysomes (Fig. 9, compare lanes 1 and 3).

DISCUSSION

The assay described here will serve as the foundation for future studies on the molecular mechanisms of protein import into glyoxysomes. Therefore, it is crucial that it be not only consistent and reproducible, but also clearly physiologically relevant. Such an in vitro import assay is available to study import into mammalian peroxisomes (Imanaka et al., 1987; Lazarow et al., 1991), but import into yeast peroxisomes has been more difficult to reconstitute successfully (Lazarow et al., 1991; Wendland and Subramani, 1993). Although several other groups have reported on in vitro import into plant peroxisomes, until recently (Behari and Baker, 1993; Horng et al., 1995) critical controls were missing or incomplete (Gietl and Hock, 1986; Mori and Nishimura, 1989; Sautter et al., 1988). We have shown that protein import into isolated pumpkin glyoxysomes was time-, temperature-, and energy-dependent, as well as saturable and competitive in nature. In addition, we showed that this process was specific for peroxisomal pro-

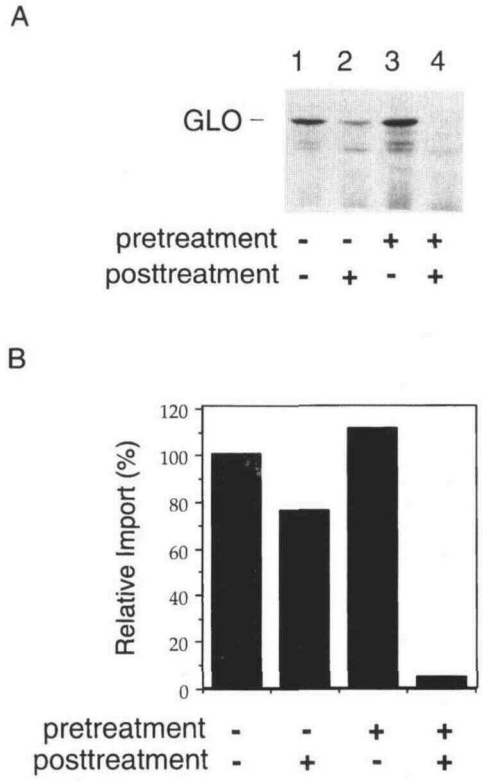

Figure 9. Protease pretreatment of isolated glyoxysomes prevents subsequent import of GLO. Pumpkin glyoxysomes were preincubated with trypsin and chymotrypsin to digest surface-exposed proteins or with import buffer as a control for 20 min at room temperature. The protease pretreatments were stopped by the addition of trypsin/chymotrypsin inhibitor. ATP and translation products were added to initiate import. Posttreatment of the import reactions with proteinase K removed any remaining external GLO, as expected; imported GLO was protease-protected. A, Autoradiograph from a representative "protease-shaving" experiment. B, Quantitated results from the experiment shown in A.

teins and inhibited by protease pretreatment of the organelles. Each of these criteria is expected of a physiologically relevant process. To our knowledge, our results represent the first detailed demonstration of the time course of protein import into glyoxysomes (Fig. 6), of the saturation of the system (Fig. 7), of the ability of unlabeled GLO to compete with radiolabeled GLO for import (Fig. 8), and of the effect of protease pretreatment of glyoxysomes on subsequent protein import into the organelles (Fig. 9). These experiments establish the relevance of this system for studying the molecular mechanisms of protein import into glyoxysomes in vitro.

One way in which the in vitro assay will be especially useful is in investigating the potential role of cytosolic factors in protein import into plant peroxisomes. This problem cannot be addressed using current standard genetic and/or in vivo approaches. Cytosolic factors may be involved in in vivo protein import into yeast and mammalian peroxisomes (Wendland and Subramani, 1993; Rachubinski and Subramani, 1995; McNew and Goodman, 1996) and, possibly, plant glyoxysomes. We did not specifically test for this possibility in the experiments reported here. However, a homolog of the bacterial molecular chaperon DnaJ protein has been identified and localized to glyoxysomal membranes from cucumber cotyledons (Preisig-Muller et al., 1994), and we have preliminary results that implicate the involvement of a cytosolic factor in peroxisomal protein import (W. Crookes and L. Olsen, unpublished data). The in vitro assay can now be used to control precisely the biochemical conditions for protein import as we begin to dissect the molecular mechanisms of this process.

Protein import into chloroplasts, mitochondria, and the ER is known to require interaction between the translocating protein and multiple receptors on the surface of the organelle (Verner and Schatz, 1988; Keegstra et al., 1989; Kessler et al., 1994; Schnell et al., 1994; Lithgow et al., 1995; Lill and Neupert, 1996). Genetic studies with yeast peroxisomes have led to the identification of a number of mutants in peroxisome assembly. A combination of complementation analyses and in vivo import assays have been used to define the roles of the mutations found (reviewed in Rachubinski and Subramani, 1995; McNew and Goodman, 1996). The results of these genetic analyses suggest that different peroxisomal proteins may be recognized by different receptors, implying that more than one peroxisomal import pathway exists. Our results provide supporting evidence that protein import into glyoxysomes also occurs via surface receptors. Import of GLO and IL into glyoxysomes was stimulated by the addition of ATP (Fig. 5 and data not shown). The import reaction was saturable and specific for peroxisomal proteins. Excess unlabeled peroxisomal proteins were able to saturate the binding and/or translocation sites on the glyoxysomes and prevent efficient import of the radiolabeled translation products (Fig. 8). In addition, protease pretreatment of the glyoxysomes with trypsin/chymotrypsin abolished their ability to subsequently import proteins (Fig. 9). Taking all of these results together, we conclude that in vitro import of proteins into glyoxysomes is receptor-mediated.

Although the protease pretreatment completely inhibited further protein translocation, it did not affect the amount of protein that bound to the glyoxysomes (Fig. 9, compare lanes 1 and 3). This suggests that the topogenic domain on the PTS1 receptor to which the PTS1 protein binds may not be susceptible to trypsin or chymotrypsin digestion. Perhaps the PTS1 receptor has one domain to which the targeting tripeptide binds and another region through which the protein is actually translocated. Alternatively, there may be more than one protein on the surface of the membrane that is involved in the binding and translocation of peroxisomal proteins. One protein may bind the PTS1, but not be susceptible to trypsin/chymotrypsin treatment, whereas another protein that is trypsin/chymotrypsin-sensitive may function for protein translocation. Protease treatment with either proteinase K or thermolysin did not affect the levels of GLO import (data not shown), providing further evidence that there may be multiple proteins or multiple pathways for protein import. In fact, Wolins and Donaldson (1994) described a receptor for protein import into glyoxysomes that had two binding sites

with different affinities for a synthetic peptide containing a PTS1 sequence.

The controversial subcellular localization of the PTS receptors has raised the question of whether the receptors bind to peroxisomal proteins in the cytosol prior to interaction with a translocation apparatus on the surface of the membrane (reviewed in Rachubinski and Subramani, 1995; McNew and Goodman, 1996). Both the putative PTS1 and PTS2 receptors have been reported to be primarily cytosolic and partially peroxisomal and even intraperoxisomal, leading to the proposal of rather complex models for peroxisomal protein import (Rachubinski and Subramani, 1995; McNew and Goodman, 1996). In these models, the PTS1 and PTS2 receptors function both in the cytosol and at the peroxisomal membrane, where additional proteins of the translocation channel actually effect transport of the targeted protein into the peroxisomal matrix. Because there was no direct addition of cytosol to the in vitro assays (a small amount of cytosolic extract was added with the translation products), import relied primarily on whatever surface receptors were present on the isolated glyoxysomes. Our results suggest that such receptors were present in sufficient quantities to support peroxisomal protein import. The efficiency of import, however, may be improved if a full complement of cytosolic and membrane-associated components are present.

In conclusion, the development of the in vitro protein import assay reported here represents a significant advancement for the study of the mechanisms of higher plant peroxisomal protein transport and provides strong evidence that proteinaceous receptors are required for glyoxysome biogenesis. Application of the powerful genetic advantages of yeast combined with the careful molecular and biochemical analyses possible with the in vitro assay should allow for rapid progress in our understanding of protein import into plant peroxisomes.

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