Characterization of intermediates in the process of plant peroxisomal protein import

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A hybrid protein in which the immunoglobulin G-binding domain of *Staphylococcus aureus* protein A replaced the N-terminal 43 amino acids of glycolate oxidase (a peroxisomal protein) was affinity purified after expression in *Escherichia coli* and used to study peroxisomal protein import *in vitro*. The fusion protein, which co-purifies with the bacterial chaperones dnaK and groEL, binds to glyoxysomes and is partially translocated in an ATP-dependent reaction which is independent of eukaryotic cytosol. Both binding and translocation are dependent upon the amount of glyoxysomes present. The partially translocated species has a transmembrane location and is extractable by salt, indicating that it is held in the membrane by ionic interactions. In the absence of ATP, the fusion protein binds to the surface of the glyoxysomes and competes the binding of authentic matrix proteins. The surface-bound protein can be chased to the transmembrane species upon the addition of ATP. These results indicate that the surface-bound form is a true translocation intermediate. The availability of this fusion protein in milligram quantities offers the possibility to use the intermediate formed in the absence of ATP and the transmembrane species to probe interactions with the peroxisome import machinery.

Keywords: glyoxysome/import intermediates/peroxisome/protein translocation

Introduction

Peroxisomes are organelles found in almost all eukaryotic cells. In plants, they contain enzymes associated with major pathways of carbon metabolism such as photorespiration, β -oxidation and the glyoxylate cycle (the latter occurs in specialized peroxisomes called glyoxysomes). New functions and enzymatic constituents of plant peroxisomes are still being discovered (Nakamura *et al.*, 1997). Recent years have seen major advances in understanding of peroxisomal protein import, mainly in animals and yeasts and to a lesser extent in plants (reviewed in Subramani, 1998). Two classes of targeting signal which function to direct proteins to the peroxisomal matrix have

been identified and characterized; a C-terminal tripeptide PTS-1 (peroxisome targeting signal 1), based on a loose consensus of the sequence serine–lysine–leucine (Gould *et al.*, 1989), and a sequence PTS-2 (peroxisome targeting signal 2), found at or near the N-terminus of the thiolases (Swinkels *et al.*, 1991) and a few other proteins. Variations upon these signals appear to be functional in all organisms, including plants (Volokita *et al.*, 1991; Hayashi *et al.*, 1996; Kato *et al.*, 1996). The receptors for these two classes of targeting signal have also been identified, Pex5p (Distel *et al.*, 1996), which recognizes the PTS-1 signal (Dodt *et al.*, 1995; Terlecky *et al.*, 1995), and Pex7p (Distel *et al.*, 1996), which binds PTS-2 (Rehling *et al.*, 1996; Zhang and Lazarow, 1996). These receptor proteins are thought to bind peroxisomal proteins in the cytosol and bring them to the membrane. The PTS-1 receptor has been shown to dock with an integral membrane protein Pex13p via a cytosolically oriented SH3 domain (Elgersma *et al.*, 1996; Erdman and Blobel, 1996; Gould *et al.*, 1996).

Subsequent events are unclear. It is known that peroxisomes can import folded proteins (Glover *et al.*, 1994; McNew and Goodman, 1994). However, the mechanism by which this occurs remains speculative. Various models have been proposed which include regulated static pores, dynamic pores which assemble when the protein and its receptor are delivered to the membrane, and even import by an endocytic-like event. At some point, the receptors must be released and recycled to interact with more peroxisomal proteins in the cytosol.

Genetic screens in several yeast species and the study of inherited human peroxisomal assembly disorders have led to the identification of a number of gene products required for peroxisomal protein import (reviewed in Subramani, 1998). Some of these gene products are predicted from their sequences to be integral membrane proteins and are therefore candidates to be components of the import machinery. At present, the biochemical functions of these proteins are understood poorly, if at all.

In other organelles, the formation of intermediates in the process of protein translocation has proved extremely fruitful in the identification and subsequent biochemical characterization of components of the protein import machinery (reviewed in Baker *et al.*, 1996). This approach has exploited the availability of *in vitro* reconstituted systems and the ability to manipulate conditions such that proteins are arrested en route into the organelle. We previously have reported *in vitro* reconstitution of the import of two plant peroxisomal proteins, isocitrate lyase (Behari and Baker, 1993) and glycolate oxidase (Hornig *et al.*, 1995), using isolated sunflower glyoxysomes and *in vitro* translated proteins. We sought, therefore, to modify the *in vitro* import assay to permit the identification and characterization of import intermediates which could be useful in future experiments aimed at isolating and/or

investigating functional interactions of an imported protein with components of the peroxisomal protein import machinery. One of the limitations of using proteins translated *in vitro* is that only very small amounts of protein are produced. This makes analysis of proteins associated with translocation intermediates very difficult, particularly if antisera to candidate proteins are not available. As an alternative, recombinant proteins purified from bacteria can be used to generate large amounts of translocation intermediates, and proteins associated with such intermediates purified.

To permit easy purification of the expressed protein from bacteria, and the re-isolation of any intermediates formed, the IgG-binding domain of protein A from *Staphylococcus aureus* was fused at the N-terminus of spinach glycolate oxidase in place of the N-terminal 43 amino acids of the native protein. Glycolate oxidase has been shown to be targeted to, and imported by, isolated glyoxysomes *in vitro*, and a mutant which lacks the first 59 amino acids at the N-terminus is also imported *in vitro* (Horng *et al.*, 1995). The protein has also been shown to possess a PTS-1 which is functional *in vivo* (Volokita, 1991). A chloroplast transit peptide fused to protein A has been used successfully to form translocation intermediates and to purify components of the chloroplast protein import machinery (Schnell *et al.*, 1994).

Results

Expression and purification of protein A–glycolate oxidase

The protein A–glycolate oxidase hybrid protein (pAGO) was purified from the soluble fraction of the *Escherichia coli* cell lysate by affinity chromatography on an immunoglobulin G–Sepharose column (Pool *et al.*, 1998), and analysed by SDS–PAGE and immunoblotting. A typical fusion protein preparation is shown in Figure 1A. The three polypeptides visible on the Coomassie-stained gel have experimentally determined mol. wts of 70.6, 57.4 and 51.2 kDa. The 70.6 kDa polypeptide cross-reacts with antibodies against *E.coli* dna K and can be removed by washing the IgG column with ATP prior to elution of the bound fusion protein (data not shown). The 57.4 kDa protein cross-reacts with antibodies against *E.coli* groEL (data not shown). The size of the pAGO fusion protein is in good agreement with the size of 53.1 kDa predicted from the sequence. The yield of protein in the fusion protein preparation was 2.1 ± 0.3 mg/l of cell culture, and the purity of different preparations (as estimated by Coomassie blue staining of gel samples) was 20–30%.

Protein A–glycolate oxidase is targeted to isolated glyoxysomes

The purified hybrid protein was incubated with glyoxysomes isolated from sunflower cotyledons. Organelle pellets from the import reactions were analysed by SDS–PAGE and immunoblotting (Figure 1B). Lane 1 is a 10% standard of the hybrid protein to allow quantification. Lanes 2 and 4 show that in the presence or absence of ATP the hybrid protein re-isolates with the organelles. Thermolysin treatment of reactions carried out in the presence of ATP (lane 3) revealed a protease-protected species ~2 kDa smaller than the hybrid protein, suggesting

Fig. 1. Import of protein A–glycolate oxidase (pAGO) into isolated sunflower glyoxysomes. (A) Left: 2.5 µg of a typical fusion protein preparation stained with Coomassie blue. Right: immunoblot of the same preparation. (B) Import reactions carried out with pAGO in the presence (lanes 2, 3, 6, 8 and 9) or absence (lanes 4 and 5) of ATP and a regenerating system. Import reactions in lanes 8 and 9 were supplemented with wheat germ lysate. Lane 7, mock import without organelles; lane 10, mock import reaction with no added hybrid protein. Reactions were incubated for 15 min at 26°C. Reactions from lanes 3, 5 and 9 were treated with thermolysin for 30 min on ice. The protease was inhibited and the glyoxysomes were re-isolated through a sucrose cushion. The organelle pellet from lane 6 was then treated with thermolysin in the presence of Triton X-100. All the organelle pellets were then analysed by SDS–PAGE and immunoblotting. Lane 1 represents 10% of the hybrid protein added to each import reaction. (C) Import of *in vitro* translated full-length glycolate oxidase and pAGO. Lane 1, 10% standard of each translation product; lane 2, import reactions in the presence of ATP and not treated with protease; lane 3, import reactions in the presence of ATP, protease treated; lane 4, import reaction in the absence of ATP, not protease treated; lane 5, import reaction in the absence of ATP, protease treated.

that a small portion of the hybrid protein was exposed to protease, consistent with incomplete translocation. Protease protection was dependent upon ATP (compare lanes 3 and 5; also Figures 4 and 6). In some experiments, such as in the one shown in Figure 1B (but not those in Figures 4 and 6), a very small amount of protease-protected pAGO is seen in the absence of additional ATP. As the organelles

are not treated with apyrase, this may reflect a low and variable amount of ATP re-isolating with the glyoxysomes, possibly bound to component(s) of the import machinery. ATP hydrolysis is required for the formation of the protease-protected species as the non-hydrolysable analogue ATP- γ -S cannot substitute for ATP and in fact inhibits the formation of this species at low ATP concentrations (Pool, 1997). In the presence of detergent and protease (Figure 1B, lane 6), the protein is degraded completely, indicating that protection is due to an intact organelle membrane. No hybrid protein re-isolated in the absence of any glyoxysomes (lane 7).

Previous studies using the *in vitro* import assay have been carried out in the presence of wheat germ lysate from the translation reaction (Behari and Baker, 1993; Horng *et al.*, 1995). To see if the partial protease protection was due to the absence of a factor normally present in the translation mix, wheat germ lysate was added as would have been present from a translation reaction. This had no effect on association (Figure 1B, lane 8) or protease protection (lane 9). Lane 10 shows a mock import reaction with no hybrid protein added. This confirmed that the signals seen on the immunoblot were due solely to cross-reaction with the hybrid protein, and not a component of the glyoxysome preparation. Thus, the hybrid protein is targeted efficiently to the organelles and shows partial protease protection in the presence of ATP. This partially protected species is not a kinetic intermediate as it cannot be chased into the fully protected form in longer incubations (data not shown). An almost identical protein A-glycolate oxidase hybrid protein (which differs by only five amino acids at the extreme N-terminus of the protein A domain) produced by *in vitro* transcription and translation in wheat germ lysate showed the same partial protection phenomenon (Figure 1C, lane 3), but authentic glycolate oxidase was completely protected (Figure 1C; and see also Horng *et al.*, 1995; Pool *et al.*, 1998 for *in vitro* import reactions with full-length glycolate oxidase). The partial protease protection therefore cannot be due to the lack of some cytosolic component(s), or due to the presence of groEL and/or dnaK in the hybrid protein preparation.

Control experiments performed with the protein A domain alone or protein A fused to a non-glyoxysomal protein (synaptotagmin) did not result in the formation of any protease-protected species (Pool, 1997).

To evaluate the organelle specificity of pAGO import, sunflower cotyledon mitochondria were prepared by differential and sucrose density gradient centrifugation according to Leaver *et al.* (1985). The gradient fractions were assayed for malate synthase activity [which serves as a marker for matrix plus glyoxysomal membranes under low salt conditions (Chapman *et al.*, 1989)] (Figure 2A) and immunoblotted for isocitrate lyase (a glyoxysomal matrix marker) and the mitochondrial adenine nucleotide translocator (Figure 2B). Intact glyoxysomes were recovered in fractions 1–5 of the gradient, well resolved from mitochondria (fractions 5–12), although the mitochondrial fractions were contaminated with glyoxysome membranes, as shown by the presence of some malate synthase activity in fractions 6–12. Fractions 6–10, which were free of isocitrate lyase immunoreactivity and therefore did not contain intact glyoxysomes, were pooled, pelleted and

Fig. 2. Protein A-glycolate oxidase is not imported by mitochondria. (A) A mitochondrial pellet prepared from 3 day sunflower seedling cotyledons was separated on a 1.6–0.75 M linear sucrose gradient. Malate synthase activity was measured in each of the fractions. (B) Fractions from the gradient shown in (A) immunoblotted with antibodies against isocitrate lyase and the mitochondrial adenine nucleotide translocator. (C) Fractions 6–10 from the gradient in (A) were pooled. The fraction was split in half and one half was diluted, pelleted and resuspended in peroxisome import buffer; the other half was diluted, pelleted and resuspended in mitochondrial import buffer. A total of 100 μ g of protein was used in each import reaction, as previously described.

resuspended in either glyoxysome import buffer or mitochondrial import buffer (Winning *et al.*, 1995). Import reactions with pAGO were then carried out using the conditions used for glyoxysomes in this study or using conditions optimal for mitochondrial protein import (Winning *et al.*, 1995). Under either conditions, ATP-independent binding was seen (Figure 2C lanes 2, 4, 8 and 10), but no protease protection could be detected (lanes 3, 5, 9 and 11). The binding is most likely due to broken glyoxysomal membranes which contaminate the

Fig. 3. Effects of organelle concentration and hybrid protein concentration on binding and import of protein A-glycolate oxidase. (A) Import reactions were carried out with purified pAGO and isolated glyoxysomes. The hybrid protein concentration was fixed at 200 nM (the concentration of pAGO is calculated based on the total protein concentration of the preparation which overestimates the amount of pAGO by a factor of 4–5) and the amount of organelles added to each reaction was titrated from 0 to 125 µg. (B) Import reactions were carried out with purified glyoxysomes and purified pAGO. The concentration of glyoxysomes was fixed at 200 µg and the concentration of hybrid protein varied between 50 and 350 nM (based on total protein). In each experiment, the organelles were re-isolated and analysed by SDS-PAGE and immunoblotting and the results quantified by scanning densitometry with two different exposures of the immunoblot.

mitochondrial fractions (Figure 2A). The hybrid protein does not associate non-specifically with membranes (Figure 3A). The results shown in Figure 2C demonstrate conclusively that the partial protease protection seen in Figure 1B is due to intact glyoxysomes.

Binding and protease protection are proportional to the glyoxysome concentration and the amount of hybrid protein used in the import assay

Import reactions were carried out with increasing amounts of isolated glyoxysomes (0–125 µg) with the concentration of pAGO fixed at 200 nM. To improve recoveries at low concentrations of glyoxysomes, 500 µg of red blood cells from calf ascites (washed and resuspended in import

buffer) were added to all the samples after the protease step but prior to re-isolation of the glyoxysomes.

Figure 3A shows the quantification of the re-isolated glyoxysome pellets. In the lanes where no glyoxysomes were added, very little hybrid protein was recovered, demonstrating that the hybrid protein does not associate non-specifically with the red blood cells and that any material which is re-isolated can be attributed solely to glyoxysome association. Both binding and protease protection increase and begin to plateau at higher concentrations of glyoxysomes. More fusion protein can be bound than can be imported. This may be because a proportion of the hybrid protein is competent for binding but not translocation. Alternatively, there may be a larger number of sites for binding than there are sites for translocation. In standard import reactions, a glyoxysome protein concentration of >100 µg was used, which gives high levels of binding and import without the need to add red blood cells in order to achieve quantitative recoveries.

In a second experiment, import reactions were performed with the amount of organelle protein fixed at 200 µg, but with varying concentrations of pAGO (Figure 3B). Both binding and import increased with increasing concentration of hybrid protein preparation in an approximately linear manner. About 75% of the bound material could be imported. Again this suggests that there are a larger number of sites for binding than there are for translocation.

The hybrid protein is present in a transmembrane location

To determine if the partial protease protection of the fusion protein was due to translocation arrest, import reactions carried out with pAGO were either treated with protease and re-isolated according to the standard protocol, or organelles were re-isolated through a sucrose cushion to remove free hybrid protein, subjected to hypotonic lysis and the membranes and adhering proteins pelleted by centrifugation. The results are shown in Figure 4A. The hybrid protein re-isolated with the organelles in the presence (lane 1) and absence (lane 6) of ATP. Partial protease protection of the hybrid protein was only seen in the presence of ATP (compare lanes 2 and 7). In both the presence (lane 3) and absence of ATP (lane 8), the hybrid protein re-isolated in the membrane fractions. No hybrid protein was seen in the supernatant fractions (lanes 5 and 10). In the presence and absence of ATP, all of the hybrid protein present in the membrane fraction was sensitive to protease (lanes 4 and 9). In the case of the hybrid protein incubated in the presence of ATP, this is only consistent with a transmembrane location. If the protein was protected from the protease by the presence of other proteins, either in an aggregate or associated with the matrix, then the protein would still be expected to be resistant to protease in the pellet fraction. If the fractionation conditions had disrupted such an aggregate, then the protein would not have been pelleted with the membranes.

In mock reactions, carried out in the absence of organelles, the hybrid protein was only present in the supernatant fraction (Figure 4B). The observed re-isolation of the hybrid protein with membranes, and loss of protease protection, is exactly as would be predicted for a transmembrane species.

Fig. 4. The partially protected species is in a transmembrane location. (A) Import reactions were carried out with isolated glyoxysomes and purified pAGO in the presence (lanes 1–5) or absence (lanes 6–10) of ATP. After the import step, whole organelles (O) were re-isolated (lanes 1 and 6) or treated with thermolysin and then re-isolated (lanes 2 and 7). Alternatively, import reactions were fractionated as described in Materials and methods. The resulting membrane pellet (P) and supernatant (S) were either analysed directly (lanes 3, 5, 8 and 10) or, in the case of the pellet fractions, after protease treatment (lanes 4 and 9). (B) Purified pAGO was ultracentrifuged under the same conditions as in (A), but in the absence of organelles. This produced a ‘pellet’ fraction (P, lane 1) and a supernatant (S, lane 3). The pellet fraction was also treated with protease (lane 2). All samples were analysed by SDS–PAGE and immunoblotting.

The hybrid protein is held in the transmembrane state by salt-sensitive interactions

To investigate the nature of the interactions holding the protein in the membrane, glyoxysomes were allowed to import the fusion protein, lysed as before, then the membranes were re-isolated and washed with a range of concentrations of sodium chloride or with alkaline sodium carbonate (Figure 5). As controls, malate synthase, a matrix protein peripherally associated with the glyoxysomal membrane (Chapman *et al.*, 1989), and a 39 kDa integral peroxisomal membrane protein (PMP) (Corpas *et al.*, 1994) were detected by immunoblotting. Most of the malate synthase is removed by washing the membranes with 0.2 M NaCl and the remainder is removed completely at 0.5 M. Treatment with alkaline sodium carbonate is also effective at completely removing malate synthase from the membrane fraction.

The hybrid protein behaves in a manner broadly similar to malate synthase. Protein bound in the absence of ATP is removed completely by 0.2 M NaCl treatment. Likewise, the majority of the transmembrane species formed in the presence of ATP can be removed by 0.2 M salt, and the remainder is removed by 0.1 M sodium carbonate. In contrast, as expected, the 39 kDa integral PMP is not extracted from the membrane by either salt or sodium carbonate treatment. Taken together, these data indicate that the hybrid protein is held in a transmembrane location by relatively weak protein–protein interactions. The pos-

Fig. 5. The partially imported protein A–glyoxylate oxidase hybrid protein is held in the membrane by salt-sensitive interactions. Import reactions were carried out with purified pAGO and isolated glyoxysomes. After import in the presence or absence of ATP, membranes were prepared and washed as described in Materials and methods and analysed by SDS–PAGE and immunoblotting with rabbit anti-bovine antibody or with polyclonal antisera raised against malate synthase and a 39 kDa integral PMP.

sibility that the protein becomes integrated into the membrane bilayer can be excluded.

In the absence of ATP, a protease-sensitive intermediate is formed which lies on the translocation pathway

In the absence of ATP, the hybrid protein binds to glyoxysomes but remains completely accessible to protease. To investigate if this species is a *bona fide* translocation intermediate, the ability of this bound form to be translocated subsequently upon the addition of ATP was investigated.

Import reactions were carried out in two stages (Figure 6); standard import reactions in the absence of ATP were re-isolated and resuspended in import buffer with or without ATP but with no additional hybrid protein. A second import incubation was then carried out and the organelles treated with protease to test for the partial translocation of the hybrid protein. In a single import step in the absence of ATP, the hybrid protein bound to the organelles (lane 1), but was not protease protected (lane 2). The organelles were competent for import as, in the presence of ATP, ~50% of the hybrid protein was protected from protease (lane 4 and Figure 6B). With two import steps both in the absence of ATP, no protease protection was observed (lane 6). In contrast, if ATP was present in the second step then protease protection was observed (lane 8). The amount of imported pAGO is considerably less (~25%) than that observed for a one-step import reaction (compare lanes 4 and 8). This is due to damage to the organelles during the re-isolation step as, when organelles incubated in the absence of hybrid protein (lanes 9 and 10) were re-isolated and then resuspended in import buffer with ATP and hybrid protein, the import efficiency was much reduced (~10%). The surface-bound material could be translocated more efficiently in the second incubation than freshly added precursor (compare lanes 8 and 10), indicating that the surface-bound material has a higher competence for import than free precursor.

Fig. 6. Surface-bound protein A–glycolate oxidase can be chased into the partially translocated state. Import reactions were carried out using purified pAGO and isolated glyoxysomes. Import was carried out in the presence (lanes 3 and 4) or absence (lanes 1, 2, 5, 6, 7 and 8) of ATP. After a first import step, reactions were either subjected to no further incubations (lanes 1–4) or were re-isolated and resuspended in import buffer with (lanes 7 and 8) or without (lanes 5 and 6) ATP. Mock reactions (lanes 9 and 10) were carried out such that the first ‘import’ incubation was in the absence of hybrid protein, the organelles were then re-isolated and resuspended in import buffer with ATP and hybrid protein and subjected to a second import step. After the final import incubations, reactions from lanes 2, 4, 6, 8 and 10 were treated with thermolysin. All reactions were then re-isolated through a sucrose pad and the pellets analysed by SDS–PAGE and immunoblotting. A standard representing 100% of the hybrid protein added to each reaction was also included (100%). The amount of hybrid protein in each pellet fraction was quantified using a scanning densitometer from three different exposures of the immunoblot. The results are displayed graphically in the upper panel.

This demonstrates that the hybrid protein bound to the surface in the absence of ATP is a true translocation intermediate as it lies on the protein translocation pathway.

As the pAGO bound under conditions of ATP depletion is a true import intermediate, it should be able to compete the binding and, therefore, the import of authentic peroxisomal proteins. Glyoxysomes were pre-incubated on ice in the absence of ATP with increasing concentrations of pAGO preparation. After 15 min pre-incubation, 2.5 μ l of *in vitro* translated spinach glycolate oxidase, watermelon glyoxysomal pre-malate dehydrogenase (MDH, Gietl, 1990) or *Arabidopsis* 22 kDa integral PMP (PMP22; H.B.Tugal, L.M.Sandalio, M.R.Pool, L.A.del Río and A.Baker, submitted) was added and the reactions incubated at 26°C for 5 min. Only 60 μ g of glyoxysomal protein was used per reaction, as this is in the linear range for both binding and import (Figure 3A), and washed red blood cells were added after the binding reaction to ensure 100% recovery of the organelles. As a control, bovine serum albumin (BSA) was added to a separate set of binding reactions at the same concentrations as the pAGO preparation. Even at 7.5 μ M (100 μ g added protein), BSA had no effect on binding of authentic glycolate oxidase to glyoxysomes (Figure 7). In contrast, addition of increasing amounts of the pAGO preparation (up to 7.5 μ M) to the

Fig. 7. Protein A–glycolate oxidase competes the binding of authentic glycolate oxidase. Glyoxysomes were pre-incubated for 15 min on ice with varying amounts of pAGO preparation (the concentration of pAGO calculated based on the total protein concentration of the preparation which overestimates the amount of pAGO by a factor of 4–5) or BSA. A 2.5 μ l aliquot of *in vitro* translated glycolate oxidase, pre-gMDH or PMP22 were added and the reactions incubated for 5 min at 26°C before re-isolation of the organelles and analysis of the pellets by SDS–PAGE and phosphorimaging. In the lower panel, the results of three separate experiments were quantified.

binding reactions resulted in ~70% reduction in binding of glycolate oxidase (Figure 7). Similar results were obtained for the binding of pre-MDH but there was little effect on the binding of PMP22. Glyoxysomes incubated with this amount of pAGO for 30 min showed >80% intactness as calculated by malate synthase latency. As only ~20% of this preparation by mass was pAGO itself, the highest concentration used corresponds to ~1.5 μ M pAGO. This is similar to the amount of unlabelled authentic glycolate oxidase required to compete import of *in vitro* translated glycolate oxidase (220 μ g/ml or 5 μ M; Brickner *et al.*, 1997) or the amount of unlabelled thylakoid proteins pLHCP and iOE23 required to compete the import of authentic proteins into isolated thylakoids (1–4 μ M; Cline *et al.*, 1993), and is nearly two orders of magnitude less than the 100 μ M concentration of synthetic peptide required to inhibit the import of urate oxidase and acyl CoA oxidase into rat liver peroxisomes (Miura *et al.*, 1992, 1994).

Discussion

A hybrid protein comprising the IgG-binding domains of *S.aureus* protein A fused to glycolate oxidase can be

expressed in bacteria and affinity purified. The purified protein can be imported efficiently by isolated glyoxysomes; up to 75% of the added protein is protease protected in an ATP-dependent manner. However, the hybrid protein is not completely protected; a 2 kDa domain remains exposed to protease. This partial protease protection is due to the hybrid protein being present in a transmembrane location, as it is completely protease sensitive in lysed organelles. The hybrid protein can be extracted from the glyoxysomal membrane by mild salt conditions, consistent with it being held by protein-protein interactions. In the absence of ATP, the hybrid protein is bound to the membrane but is completely accessible to protease. This species is a true translocation intermediate as it competes the binding of authentic peroxisome matrix proteins (PMPs) and can be chased from the membrane-associated state into the partially protected form by the addition of ATP. Interestingly, the fusion protein competes for the binding of both a PTS-1-targeted protein (glycolate oxidase, Volokita, 1991) and a PTS-2-targeted protein (MDH, Gietl *et al.*, 1994) but not for an integral membrane protein (PMP22; H.B.Tugal, L.M.Sandalio, M.R.Pool, L.A.del Río and A.Baker, submitted). These results indicate that in the absence of ATP the fusion protein translocation intermediate is located at a point common to both matrix import pathways. Genetic evidence also supports the idea that both import pathways converge, as the only mutations which are specific for the PTS-1 and PTS-2 pathways are *pex5* and *pex7*, the receptors for the two PTSs. Pex14p interacts with both receptors and, when mutated, prevents the import of both PTS-1 and PTS-2 proteins (Albertini *et al.*, 1997; Brocard *et al.*, 1997). The lack of effect on the binding of the integral membrane protein PMP22 is consistent with findings that mutants in most peroxins do not affect insertion of membrane proteins (Subramani, 1998), indicating that they are inserted by a distinct pathway. It also allows us to exclude that high concentrations of pAGO simply coat the glyoxysome membrane and inhibit binding non-specifically.

Requirement of cytosolic factors for import

The hybrid protein can bind to the glyoxysomal membrane and be partially translocated in the absence of any eukaryotic cytosol. The only additional factors present are the bacterial chaperones dnaK (an Hsp70-type chaperone which can be removed from the preparation without loss of import competence) and groEL. Studies of peroxisome protein import in permeabilized or microinjected mammalian cells have shown a requirement for cytosol (Wendland and Subramani, 1993; Rapp *et al.*, 1993). There is some evidence for a function for Hsp70 class molecules in peroxisomal protein translocation (Walton *et al.*, 1994). The role of these proteins is unclear; since unfolding is not a pre-requisite for import, they would probably not be required to maintain the protein in an unfolded conformation. Alternatively, they may be required to maintain the PTS in a suitable conformation to be recognized by the PTS receptor. If cytosolic chaperones are required, this study would suggest that their role can be complemented by chaperones still bound to the surface of the glyoxysomes, or by groEL. Both an Hsp70-type chaperone (Corpas and Trelease, 1997) and a dnaJ homologue

(Preisig-Müller *et al.*, 1994) have been found associated with plant glyoxysomal membranes.

A cytosolic PTS-1 receptor is not required for import of the pAGO hybrid. It may be that the cytosolic location of the receptor is more crucial *in vivo* and may promote transport of the protein to the peroxisome. *In vitro*, almost the only membranes present are glyoxysomal membranes and they are probably present at a much higher concentration than *in vivo*. Either under these conditions the requirement for Pex5p is circumvented, or there may be sufficient receptor associated with the isolated glyoxysomes to mediate import. The conditions used to isolate glyoxysomes for the *in vitro* import assay, namely low temperature in the absence of ATP, have been shown to enhance the binding of the human PTS-1 receptor to the cytosolic face of the peroxisome membrane (Dodt and Gould, 1996). Studies investigating the binding of PTS-1-containing peptides to alkali-stripped castor bean glyoxysomal membranes (which should remove the PTS1 receptor) showed the presence of two different binding sites (Wolins and Donaldson, 1994). This suggests that there may be other PTS-1-binding proteins in the glyoxysome membrane in addition to Pex5p.

Why is the hybrid protein only partially translocated?

The evidence points strongly to the hybrid protein being partially translocated, but why is the translocation process not completed? As folded proteins can be imported into peroxisomes, it is unlikely that the folded conformation of the protein prevents import. The protein does not enter the lipid bilayer as it is extracted by alkaline sodium carbonate, whereas the integral 39 kDa peroxisome membrane protein is not. The observation that so much of the protein is protected from the protease in intact glyoxysomes, yet is sensitive in lysed organelles, makes it unlikely that the protein could be a peripheral membrane protein on the cytosolic face of the membrane, or that what is being observed is an ATP- and groEL-mediated refolding reaction. The most likely explanation consistent with all the data is that the 2 kDa portion of the protein is close to the translocation site such that it can be accessed by the protease. The protein essentially may have been translocated but could be unable to dissociate from the translocation machinery. This 'translocation arrest' would appear to be a property of the protein A domain, not of the bacterial chaperones associated with the fusion protein, as *in vitro* translated pAGO, but not authentic glycolate oxidase, shows the same partial translocation (Figure 1C). Partial protease protection of imported proteins has also been reported *in vivo* in the *pex5* (*pay32*) mutant of the methylotropic yeast *Yarrowia lipolytica*. In this peroxisome assembly mutant, some PTS-1-containing proteins are partially translocated with the C-terminus inside the organelle (Szilard *et al.*, 1995), highly reminiscent of the situation with the hybrid protein.

This study has shown that the pAGO fusion protein can be used to form at least one, possibly two, distinct translocation intermediates representing different steps in the pathway of protein import into glyoxysomes. A model showing the possible disposition of these two species is presented in Figure 8. The surface-bound species is shown interacting with PTS-1 receptor pre-docked on the

Fig. 8. Model showing the possible relationship between the surface-bound and partially translocated protein A-glyoxylate oxidase species.

membrane. This could prevent interaction of incoming PTS-2 proteins with Pex14p or other common components of the import machinery. Alternatively, pAGO may have progressed beyond interaction with Pex5p. Antibodies which recognize sunflower Pex5p would be required to differentiate between these models. In the presence of ATP, the hybrid protein is transferred to the translocation machinery, where it is unable to complete translocation and a small portion remains accessible to externally added protease. This model envisages the translocation complex as a pore which opens to accommodate the imported protein; however, the data do not allow any distinction to be made between this and other models which have been proposed by others (McNew and Goodman, 1996; Rachubinski and Subramani, 1996). The ability to purify the hybrid protein in chemical quantities and to re-isolate it from complex protein mixtures by virtue of the affinity of the protein A domain for IgG makes it an attractive tool for probing interactions with the glyoxysome import machinery, either by protein cross-linking or by the solubilization of native complexes containing the hybrid protein. Such experiments should give important insights into the nature of the glyoxysome protein import site and may provide biochemical evidence for the role in protein import of some of the peroxin proteins identified in other organisms by genetic methods.

Materials and methods

Protein A-glyoxylate oxidase fusion proteins

Expression and purification of the protein A-glyoxylate oxidase hybrid (pAGO1) has been described (Pool *et al.*, 1998). To produce an *in vitro* transcribed version of the fusion protein, a 1.7 kb *Hind*III fragment containing the entire reading frame with the exception of the first six amino acids was inserted into the *Hind*III site of a Bluescript SK derivative into which a start codon had been introduced by a linker insertion between the *Eco*RI and *Hind*III sites of the polylinker. This produced pAGO2, which has the N-terminal sequence MGSLKD... The pAGO1 sequence is MTMITPSLKD... This new reading frame was then re-cloned into Bluescript KS to bring it under the control of the T7 promoter to produce pAGO3.

Import of bacterially expressed protein A chimaeric proteins into peroxisomes

Isolation of glyoxysomes and import reactions were performed as described (Behari and Baker, 1993) modified according to Horng *et al.* (1995). Isolated glyoxysomes routinely were observed to be between 60

and 85% intact, estimated on the basis of latency of malate synthase. Import reactions using purified protein A chimaeric proteins were performed as described previously (Behari and Baker, 1993) but with the following modifications. Unless otherwise stated, 150 µg of glyoxysomal protein was incubated with 100–500 ng of purified protein A hybrid protein preparation (added in a volume of 10 µl). Reactions carried out in the presence of an energy-regenerating system contained 160 mM creatine phosphate, 1.25 mg/ml creatine phosphokinase and 1 mM ATP. Reactions carried out in the absence of ATP and the regenerating system were not treated with apyrase. After the import incubation and protease treatment, organelles were re-isolated and then analysed by SDS-PAGE and immunoblotting. pAGO was detected with horseradish peroxidase-conjugated rabbit anti-bovine antisera (Sigma) at a working dilution of 1:5000–1:10 000.

For competition experiments, glyoxysomes (60 µg) were pre-incubated on ice in the absence of ATP with the indicated amount of fusion protein preparation or BSA for 15 min. *In vitro* translated protein (2.5 µl) was added and the reaction placed at 26°C for 5 min. Washed red blood cells (500 µg) were added and the organelles re-isolated through a sucrose cushion and analysed by SDS-PAGE and phosphoimaging.

Preparation of mitochondria and import reactions performed under mitochondrial import conditions were carried out according to Leaver *et al.* (1985) and Winning *et al.* (1995).

Post-import fractionation of glyoxysomes

Import reactions with pAGO carried out in the presence or absence of ATP and not treated with protease were re-isolated through a sucrose pad. The organelles were resuspended in 900 µl of 25 mM HEPES-KOH pH 7.2, transferred to a hand homogenizer (Jencons) and lysed with 10 strokes, the volume made up to 1 ml and 250 mM KCl and the lysate homogenized again. The lysate was centrifuged for 20 min at 256 000 g in a Beckman TL100.3 rotor at 4°C. The pellet was resuspended in import buffer (buffer B, Behari and Baker, 1993). Protease treatment of the pellet fractions was carried out under identical conditions as those for whole organelles. For salt washing of membranes, organelles were lysed as above except KCl was omitted. Membrane pellets were washed by homogenization in the required salt concentration or 0.1 M sodium carbonate pH 11.5 and recentrifugation.

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