Peroxisomes are nearly ubiquitous, single membrane-bound organelles that are responsible for the β-oxidation of fatty acids and for hydrogen peroxide degradation (1). Higher plants possess several classes of peroxisomes, which are distinguished by the metabolic enzymes that constitute the peroxisomal matrix and for hydrogen peroxide degradation (1). Peroxisomal proteins are synthesized in the cytoplasm and post-translationally translocated into the organelle. The role of chaperones and protein folding in peroxisomal protein transport is still unclear. Translocation of proteins into mitochondria requires that precursor proteins assume an extended conformation; cytosolic chaperones are thought to help maintain this conformation. In contrast, peroxisomal protein import does not require unfolding of the targeted protein. However, the molecular chaperones Hsp70 and Hsp40 may be important for translocation. We present several lines of evidence that show that plant peroxisomal protein import is enhanced by chaperones. First, peroxisomes isolated from heat-shocked pumpkin seedling tissues exhibited increased protein import relative to control peroxisomes. Second, antibodies raised against wheat germ cytosolic Hsp70 and *Escherichia coli* Hsp90 inhibited import of the peroxisomal protein isocitrate lyase. To our knowledge, this is the first time that Hsp90 has been directly implicated in a protein transport event. Third, peroxisomal proteins were immunoprecipitated by wheat germ Hsp70 antibodies. We also present results that suggest that the efficiency of peroxisomal protein import is influenced by the structure of the targeted protein; monomeric isocitrate lyase was imported more efficiently than oligomeric isocitrate lyase. Taken together, these data demonstrate that the assembly state of peroxisomal proteins and the chaperones that may mediate those states are both important for efficient peroxisomal protein import.

Peroxisomes lack an organellar genome. Therefore, all matrix peroxisomal proteins are nuclear encoded, translated on free cytosolic ribosomes, and post-translationally translocated into the organelle. They are directed to the organelle by a peroxisomal targeting signal (PTS) present either at the carboxyl terminus (PTS1) (4–8) or at the amino terminus (PTS2) of the protein (9, 10). Translocation of PTS1- and PTS2-containing proteins probably relies upon common components as well as components unique to each type of PTS (reviewed in Refs. 11 and 52). Some of the PTS-specific pathway components have been shown to bind the PTS signal sequences and have been localized to the cytoplasm (12–17). Two of these are Pex5p, a protein that recognizes PTS1 signal sequences, and Pex7p, which recognizes the PTS2 sequence (12–17). It is thought that these proteins may be cytoplasmic receptors that accompany peroxisomal proteins to the membrane.

Cytoplasmic chaperones have been implicated in protein transport across many cellular membranes including those of mitochondria, plastids, nuclei, and the endoplasmic reticulum (reviewed in Refs. 18, 19, and 49). Chaperones such as Hsp70 and Hsp90 control protein folding reactions throughout the cell. Hsp70s bind newly synthesized proteins to support proper folding and prevent nonspecific aggregation reactions (19, 49). This interaction is dependent upon nucleotides and co-chaperones such as Hsp40 (20). Hsp70 is also part of a “superchaperone complex,” which, together with Hsp90 and several other components, controls the maturation of kinases and the activation of steroid receptors (21–24).

Chaperones and/or other cytosolic factors may play a role in peroxisomal protein transport. Import into mammalian peroxisomes is dependent upon the presence of cytosol; the necessary cytosolic factors are most likely proteinaceous, since they are heat-labile (25, 26). In addition, an Hsp70 colocalizes with PTS-containing proteins and with peroxisomal membranes, and antibodies directed against bovine cytosolic Hsp73 inhibit peroxisomal protein import (27). Finally, an Hsp40 and an Hsp70 have been localized to the glyoxysomal membrane (28, 29); an Hsp70 has recently been identified in the matrix of glyoxysomes (50).

Peroxisomal protein import does not require unfolding of the targeted protein (30–34). Hausler *et al.* (31) showed that a DHFR fusion protein stabilized in its native folding state by aminopterin gains access to the matrix of trypanosome glycosomes. Subramani and co-workers (30) demonstrated that 9-nm gold particles could be imported into mammalian peroxisomes. In addition, “piggy-backing” experiments in which epitope-tagged protein constructs lacking a PTS were coexpressed with wild-type peroxisomal proteins (possessing a PTS) suggest that peroxisomal proteins assemble in the cytosol and perhaps can be imported as oligomers into *Saccharomyces cerevisiae* peroxisomes (32, 33) and into tobacco glyoxysomes (34). However, import of these large complexes into peroxisomes may have been inefficient; import was assayed only after

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lengthy incubation periods. Import is quantifiable after only 5 min in vitro and in vivo (35, 36).

We developed an in vitro protein import assay to determine the molecular mechanisms of peroxisomal protein import (36, 53). This assay permits independent manipulation of the biochemical characteristics of the organelle, peroxisomal proteins, and cytosolic factors provided by the wheat germ lysate used for protein synthesis. Isocitrate lyase is a 248-kDa homotetrameric enzyme (37) of the glyoxylate cycle; it catalyzes the conversion of isocitrate to glyoxylate in the glyoxysomes of germinating seedlings. In this study, we used the in vitro assay to investigate the role of protein folding in peroxisomal protein import in two ways: 1) indirectly, by analyzing the effects of chaperone enrichment (through heat shock) and chaperone depletion (through immunodepletion) on the import of isocitrate lyase into isolated glyoxysomes, and 2) directly, by assessing the impact of the assembly state of isocitrate lyase on its import efficiency. Our results demonstrate that the folding and/or assembly state of isocitrate lyase is important for its efficient transport across the peroxisomal membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Redi-vue [35S]methionine (specific activity, 43.5 TBq/mmol) was purchased from Amersham Pharmacia Biotech. Pumpkin seeds (*Cucurbita pepo*, var. Half Moon) were purchased from Petoseed Co., Inc. (Saticoy, CA). Wheat germ Hsp70 antisera was a generous gift from Dr. Kenneth Keegstra (Michigan State University, East Lansing, MI). *Escherichia coli* Hsp90 ([Hspg]) antisera was generated by Dr. Michael Ehrmann (University of Konstanz) and generously provided by Dr. Ursula Jakob (University of Michigan, Ann Arbor).

**Preparation of Radiolabeled Proteins**—The plasmid pILZf, containing a cDNA insert of the entire coding region of the peroxisomal enzyme isocitrate lyase in the pGEMZf(+) vector (Promega), was linearized with *BglII* or *SmaI* and transcribed with SP6 RNA polymerase as described by Brickner et al. (36). Radiolabeled isocitrate lyase was synthesized in a cell-free system in the presence of [35S]methionine. All translations were initiated by the addition of radiolabeled isocitrate lyase (36, 51).

**Isolation of Pumpkin Glyoxysomes**—Pumpkin seedlings were grown in moist vermiculite for 5–7 days in the dark at 25–28 °C. For experiments using heat-shocked tissue, dark-grown pumpkin seedlings were incubated in the dark at 40 °C for 1–3 h. Following heat shock, the cotyledons were harvested immediately for glyoxysome isolation (36, 53).

**Isolation of Glyoxysomal Proteins**—Glyoxysomal proteins were isolated as described previously (36). The glyoxysomal gel band was resuspended in isolation buffer (10 mM Hepes-KOH, pH 7.2, 1 mM EDTA, 0.3 mM mannitol, and 0.1 μg/ml each antipain and leupeptin) to a final concentration of 30–60 mg/ml total protein.

**Import Reactions and Analysis**—Standard in vitro protein import reactions were initiated by the addition of radiolabeled isocitrate lyase (0.5–10 × 10⁶ trichloroacetic acid-precipitable counts) to 300–600 μg of glyoxysomes in the presence of import buffer (25 mM Mes-KOH, pH 6.0, 0.5 mM succrose, 10 mM KCl, 1 mM MgCl₂, 5 mM MgATP, and 0.1 μg/ml each antipain and leupeptin) in a final volume of 200 μl. After a 30-min incubation, the glyoxysomes were protease-treated, repurified on a sucrose cushion, solubilized in SDS-PAGE buffer, and subjected to SDS-PAGE as described previously (36, 53). Note that only proteins protected by an intact glyoxysomal membrane would be protease-resistant. Lysed or compromised organelles would not be recovered from the sucrose cushion. Previous control studies established that protein aggregation could not account for the observed protease-protected proteins (36, 51). The percentage of relative import was determined by phospho-imaging analysis (Bio-Rad) or by excision, solubilization, and scintillation counter quantitation of the radiolabeled full-length isocitrate lyase gel band as described previously (36, 53).

Translation reactions performed in the presence of antisera (Fig. 2) resulted in varying levels of translation efficiency, so different volumes of translation products (always equivalent to 0.5 × 10⁶ trichloroacetic acid-precipitable translation counts) were added to import reactions. Translation mixture (containing all components except mRNA) were added to the protein import reactions so that the concentration of cytosolic factors (present in the wheat germ lysate) and total counts of translation products were the same for all import samples.

The native protein structure of isocitrate lyase in the translations was determined by non-denaturating PAGE on 6% polyacrylamide (pH 8.9) gels prepared using standard PAGE buffers without SDS. Gels were fixed with 10% acetic acid and stained with silver nitrate. Molecular weights were estimated by comparison of the sample lanes on the autoradiographs with a lane of Coomassie-stained high molecular weight markers (Amersham Pharmacia Biotech) from the same gel. Additionally, the heme molecule (68 kDa) present in the rabbit reticulocyte lysate in the translation reactions served as a visible internal molecular weight marker. The 62-kDa (monomer), 124-kDa (dimer), and 248-kDa (tetramer) bands were quantified by phospho-imaging analysis.

**Immunoblot Analysis**—Total protein in heat-shocked and control glyoxysomes from standard organellar preparations was measured by the BCA protein assay (Pierce). Samples were resolved by SDS-PAGE; 80 μg total protein was loaded per lane. Proteins on the resulting gel were transferred onto Immobilon PVDF transfer membrane (Millipore Corp.) in a semidry transblot apparatus (Bio-Rad). Blots were blocked with 5% nonfat dry milk in TBS-Tween (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and incubated with antisera in TBS-Tween only. Horseradish peroxidase-conjugated secondary antibodies and chemiluminescent reagents (ECL; Amersham Pharmacia Biotech) were used to visualize the results.

**RESULTS**

**Heat Shock Leads to Increased Peroxisomal Protein Import**—To examine the role of chaperones in peroxisomal protein import, we tested the import competence of glyoxysomes isolated from heat-shocked pumpkin seedlings. Chaperones Hsp70 and Hsp90 were among the proteins identified whose expression is up-regulated during the heat shock response (39). Hsp70 and Hsp40 have been localized to peroxisomal membranes (27–29), and the amount of Hsp40 associated with these membranes increases after heat stress (28). An Hsp70 has also recently been identified in the matrix of watermelon glyoxysomes (50). Thus, we hypothesized that if any of these chaperones are involved in peroxisomal protein import, glyoxysomes from heat-stressed tissues might show enhanced import capabilities compared with glyoxysomes from control tissues.

Pumpkin seedlings were heat-shocked at 40 °C for 1, 2, or 3 h. As shown in Fig. 1A, glyoxysomes isolated from 1–3 h heat-shocked tissues exhibited a 30–80% increase in the import of isocitrate lyase (assessed as protease-protected protein) when compared with protein import into glyoxysomes from control tissues. When the plants were heat-shocked for 1 h and then allowed to recover at 25–28 °C for approximately 18 h, protein import levels were approximately 3 times greater than those observed in control. Glyoxysomes, isolated from heat-shocked or control tissues, that were no longer intact were not recovered from the final sucrose cushion. In addition, isocitrate lyase in organelles with compromised membranes would be protease-accessible; the results presented represent only protease-protected (imported) protein. These results indicated that heat shock-related proteins might indeed affect the import of proteins into glyoxysomes.

To test whether the increased levels of protein import were correlated with increased levels of chaperones associated with
either harvested immediately for organelle isolation (1H R transferred to a 40 °C incubator for 1, 2, or 3 h. The tissue was then
lings were grown in the dark for 5–7 days at 28–30 °C and then
creased amounts of glyoxysome-associated Hsp70.
ited increased import competence, which correlated with in-
trate lyase) in the reaction containing glyoxysomes from tissues that
cedures.” Import samples were resolved by SDS-PAGE and quantified
by scintillation counting. The level of import (protease-protected isoci-
import reactions using glyoxysomes isolated from heat-shocked
tissues. Import reactions were initiated by the addition of isolated
glyoxysomes to import reactions containing 5 mM ATP and radiolabeled
glyoxysomal Hsp70 and performed as described under “Experimental Pro-
to the isolated glyoxysomes. A, import of isocitrate lyase synthesized in
the presence of antibodies directed against wheat germ Hsp70. (Fig. 1
showed that the amount of Hsp70 detected in these samples increased with increasing
duration of heat shock. Hsp70 levels were greatest in the
isolated glyoxysomes from tissue that had been subjected to 1 h
heat shock followed by 18 h of recovery; these glyoxysomes also
exhibited the highest level of isocitrate lyase import (Fig. 1A).
No attempt was made to further localize this Hsp70. Hsp70 has
been reported on cucumber peroxisomal membranes (29) and in
the matrix of watermelon glyoxysomes (50).

Peroxisomal Protein Import Is Decreased in the Presence of Hsp70 and Hsp90 Antibodies—To further test the role of chaperones in peroxisomal protein import, we assayed the import of
isocitrate lyase in the presence of polyclonal antibodies raised against wheat germ cytosolic Hsp70 and E. coli Hsp90. Radiolabeled translation products were synthesized in cell-free wheat germ lysate; this lysate is enriched in chaperones.2 Pero-

Fig. 1. Glyoxysomes isolated from heat-shocked tissues exhib-
ited increased import competence, which correlated with in-
creased amounts of glyoxysome-associated Hsp70. Pumpkin seed-
lings were grown in the dark for 5–7 days at 28–30 °C and then transferred to a 40 °C incubator for 1, 2, or 3 h. The tissue was then
either harvested immediately for organelle isolation (1H R transferred to a 40 °C incubator for 1, 2, or 3 h. The tissue was then
returned to 28–30 °C for approximately 18 h (1 HR + 18 HR). A, in vitro import reactions using glyoxysomes isolated from heat-shocked
tissues. Import reactions were initiated by the addition of isolated
glyoxysomes to import reactions containing 5 mM ATP and radiolabeled
isocitrate lyase and performed as described under “Experimental Procedures.” Import samples were resolved by SDS-PAGE and quantified
by scintillation counting. The level of import (protease-protected isocitrate lyase) in the reaction containing glyoxysomes from tissues that
were not subjected to heat shock (CONTROL) was set at “100% relative import”; this represents approximately 5–10% of the protein presented
in vitro to the isolated glyoxysomes. B, immunoblot analysis of the glyoxysomal extracts used for the import reactions in A, using polyclonal wheat germ Hsp70 antibodies. 80 μg of total protein was loaded in each lane.

Fig. 2. Import competence of isocitrate lyase was decreased when translation products were synthesized in the presence of Hsp antibodies. Radiolabeled isocitrate lyase was synthesized in the presence of increasing concentrations of total protein in preimmune serum or antiserum. Translations were quantified by trichloroacetic acid precipitation; radiolabeled isocitrate lyase equivalent to 0.5 × 10⁶ cpm was added to standard import reactions. In vitro protein import reactions and subsequent SDS-PAGE, autoradiography, and quantitation were performed as described under “Experimental Procedures.” The level of import in the control reaction (no antibodies) was set at “100% relative import” and corresponds to 5% of the protein presented
to the isolated glyoxysomes. A, import of isocitrate lyase synthesized in
the presence of antibodies directed against wheat germ Hsp70 (closed circles) or preimmune wheat germ Hsp70 serum (open circles). Data are from a representative experiment, which was repeated three times. B, import of isocitrate lyase synthesized in the presence of antibodies raised against cytosolic Hsp90 (closed squares) or preimmune Hsp90 serum (open squares). Data are from a representative experiment, which was repeated three times.

the peroxisomes, we performed immunoblot analysis of isolated
glyoxysomes from different heat shock treatments using antibo-
dies directed against Hsp70. Fig. 1B shows that the amount of
Hsp70 detected in these samples increased with increasing
duration of heat shock. Hsp70 levels were greatest in the
glyoxysomes isolated from tissue that had been subjected to 1 h
heat shock followed by 18 h of recovery; these glyoxysomes also
exhibited the highest level of isocitrate lyase import (Fig. 1A).
No attempt was made to further localize this Hsp70. Hsp70 has
been reported on cucumber peroxisomal membranes (29) and in
the matrix of watermelon glyoxysomes (50).

Peroxisomal Protein Import Is Decreased in the Presence of Hsp70 and Hsp90 Antibodies—To further test the role of chaperones in peroxisomal protein import, we assayed the import of
isocitrate lyase in the presence of polyclonal antibodies raised against wheat germ cytosolic Hsp70 and E. coli Hsp90. Radiolabeled translation products were synthesized in cell-free wheat germ lysate; this lysate is enriched in chaperones.2 Pero-

2 W. Crookes, unpublished results.


![Figure 4](http://www.jbc.org/)  
**FIG. 4.** Immunoprecipitation of peroxisomal isocitrate lyase (IL) using Hsp70 antibodies. Radiolabeled isocitrate lyase synthesized in a cell-free wheat germ lysate system was incubated with antisera and then precipitated onto protein A-Sepharose beads. Preimmune isocitrate lyase serum (lane 1), polyclonal antibodies against isocitrate lyase (lane 2), and preimmune wheat germ Hsp70 serum (lane 3) were used as controls. Lane 4 shows that polyclonal antibodies directed against wheat germ cytosolic Hsp70 can be used to immunoprecipitate isocitrate lyase. Lane 5 shows that the interaction is very weak in the presence of ATP.

Of the relationship between the assembly state of isocitrate lyase and import efficiency, we asked whether import efficiency was dependent upon subunit concentration. Specifically, we predicted that if monomeric isocitrate lyase is imported preferentially over tetrameric forms of the protein, low protein concentrations should favor peroxisomal protein import.

Radiolabeled isocitrate lyase was synthesized as usual except that aliquots of the translation reaction were removed at specific time points. Because the concentration of radiolabeled isocitrate lyase in the translation reaction increased, aliquots from successively later time points contained higher concentrations of isocitrate lyase than aliquots from early time points (Fig. 5A).

Nondenaturing PAGE analysis of radiolabeled isocitrate lyase from each time point confirmed the assembly state of the translation products (Fig. 5B). The active form of isocitrate lyase is a 248-kDa homotetramer (37). As expected, tetramer formation was dependent upon the concentration of the translation products; radiolabeled isocitrate lyase from early time points (lower concentrations) was predominantly monomeric, while aliquots from later time points (higher concentrations) contained predominantly oligomeric isocitrate lyase (Figs. 5B and 6A). The monomer was visible on native PAGE in the translation aliquot taken at 5 min, but the tetramer was not visible until the 12-min time point (Fig. 5B, compare lanes 1 and 4). Quantitation of the native gel shown in Fig. 5B verifies that tetramers were detectable at low protein concentrations, but isocitrate lyase was only 50% tetrameric at low concentrations, while it was 95% tetrameric at higher protein concentrations (Fig. 6A).

Next, radiolabeled isocitrate lyase from each translation time point was used as a substrate for *in vitro* import reactions. Translation products and import samples were subjected to SDS-PAGE. "Import efficiency" was calculated as the proportion of radiolabeled isocitrate lyase translation products that were protease-protected by the glyoxysomal membrane compared with the amount of full-length isocitrate lyase presented to the glyoxysomes. We found that 40–50% of the isocitrate lyase from less concentrated translation reactions was imported, while only 5–10% of translation products from the more concentrated translation reactions (Fig. 6B) were protease-protected. Therefore, translation products that were predominantly monomeric exhibited higher import efficiencies; radiolabeled isocitrate lyase that was primarily oligomeric imported less efficiently. The decrease in import efficiency with increasing isocitrate lyase concentration was not due to the prevalence of import-incompetent, super-aggregated states of isocitrate lyase; high molecular weight aggregates were not detected on native PAGE (Fig. 5B). These results indicate that the assembly state of the targeted peroxisomal protein affects its import competence. Although tetrameric isocitrate lyase was a sub-
strate for transport, monomeric isocitrate lyase was imported much more efficiently.

**DISCUSSION**

We used an established in vitro protein import assay (36, 51, 53) to investigate the influence of chaperones and protein assembly on protein transport across glyoxysomal membranes. Many researchers have reported that the folding state of peroxisomal proteins is not critical for membrane translocation (30–34). On the other hand, chaperones, which are known to control protein folding reactions, have been implicated in peroxisomal protein import (27–29). To resolve this apparent conflict, we examined the participation of Hsps in protein import, the interactions between cytoplasmic Hsps and peroxisomal proteins, and the effect of the assembly state of the targeted protein on import efficiency.

**Involvement of Glyoxysome-associated Chaperones in Peroxisomal Protein Import**—Our experiments using glyoxysomes isolated from heat-shocked tissues suggest the involvement of organelle-associated chaperones in peroxisomal protein import (Fig. 1). The direct correlation between the increase in the concentration of the glyoxysomal Hsp70 signal on immunoblots (Fig. 1B) and increased import by the organelles (Fig. 1A) suggested that heat stress altered the concentration of chaperones inside the organelle or associated with the organelle membrane and that these chaperones may participate in import. This does not necessarily mean that the Hsp70 is involved exclusively in protein folding. In fact, it is possible that Hsp70 may function directly in protein transport across the peroxisomal membrane, either as a receptor or as a transport motor, as is the case with chloroplasts and mitochondria (49, 54, 55).

The subcellular localization of the heat shock proteins that influenced import in these experiments was not investigated. The purpose of this experiment was to determine whether heat shock affected import rather than to study the specific factors involved. Other researchers, however, have localized proteins...
Involvement of Cytosolic Chaperones in Peroxisomal Protein Import—We present the first evidence of a direct interaction between cytotoxic Hsp70 and the peroxisomal matrix proteins isocitrate lyase (Fig. 4) and glycolate oxidase (data not shown). Antibodies against Hsp70 immunoprecipitated isocitrate lyase (Fig. 4) and inhibited its import into peroxisomes (Fig. 2A). Translation products synthesized in the presence of Hsp70 antibodies exhibited decreased import competence, suggesting that Hsp70 is critical for efficient import (Fig. 2A). When Hsp70 antisera was added to isocitrate lyase translation products after their synthesis, virtually no import inhibition was observed (Fig. 3A). In addition, pretreatment of the isolated glyoxysomes with antibodies against Hsp70 had no effect on subsequent isocitrate lyase import (data not shown), confirming the cytosolic location of this Hsp70 activity. Therefore, immunodepletion of cytotoxic Hsp70 during translation was more detrimental to the proteins’ import competence than removal of Hsp70 after isocitrate lyase synthesis was complete. Thus, cytotoxic Hsp70 probably acts early in the protein import pathway, i.e., during translation. It is also possible that isocitrate lyase misfolds or aggregates during translation in the absence of Hsp70 and then is no longer competent for import into peroxisomes.

One role for cytotoxic Hsp70 in peroxisomal protein import could be in maintaining the targeted protein in an import-competent state. This is the proposed role for cytotoxic Hsp70 in mitochondrial and chloroplastic protein import (reviewed in Refs. 41–43). In these import pathways, early interactions between an Hsp70 and the precursor maintain the protein in an import-competent, extended conformation, presumably so that it can bind to or move through the translocation machinery (41, 43). This model is supported by evidence that partial precursor unfolding is required for efficient mitochondrial and chloroplastic protein import (reviewed in Ref. 44).

While Hsp70 appears to function early in the peroxisomal protein import pathway, Hsp90 probably affects later steps. Isocitrate lyase import was significantly reduced when translation products were synthesized in the presence of Hsp90 antiserum (Fig. 2B) and when the antiserum was added to import reactions post-translationally (Fig. 3B). This implies that Hsp90 influences later steps in the import pathway. At this point, we cannot rule out the possibility that Hsp90 interacts with early folding intermediates as well. In other systems, Hsp90 is thought to play a role in a “superchaperone complex” with Hsp70 and several other components (22, 23). Perhaps Hsp70 and Hsp90 act in concert to maintain isocitrate lyase’s import competence.

Alternatively, Hsp90 might not interact directly with isocitrate lyase; it may instead interact with another component of the pathway that in turn interacts with isocitrate lyase. In mammalian cells, Hsp90 binding dictates the folding state of the glucocorticoid receptor (21–23). In this capacity, it appears to “prime” the receptor for interactions. Hsp90 could similarly prime the cytotoxic PTS1 or PTS2 receptors for interaction with peroxisomal proteins. Hsp90 could play an additional role, in vivo, in protein trafficking along the cytoskeleton; Hsp90 co-precipitates with actin and tubulin, suggesting that it carries its protein cargo along cytoskeletal highways (reviewed in Ref. 23). Indeed, Hsp90 binds pp60v-src kinase after its synthesis and remains complexed as the kinase travels to the cell membrane (23, 24). To our knowledge, ours is the first demonstration of an involvement of Hsp90 in a protein transport pathway.

An interaction between Hsp90 and peroxisomal isocitrate lyase was not detectable via immunoprecipitation (data not shown). Although this could mean that Hsp90 does not interact directly with isocitrate lyase, we cannot rule out the possibility that the Hsp90-isocitrate lyase interaction may be too transient to detect by immunoprecipitation.

Influence of Folding State on the Import Competence of the Targeted Protein—The observation that Hsps are involved in peroxisomal protein import suggests that peroxisomal proteins that are less folded are more import competent. To address the question of protein folding requirements for import, we designed time course experiments to manipulate the assembly state of the translation products prior to import. We found that a much greater proportion of isocitrate lyase translation products was imported at lower protein concentrations, when isocitrate lyase was predominantly monomeric (Figs. 5B and 6A).

The decrease in efficiency of import at higher isocitrate lyase concentrations was not due to a saturation effect. The concentration of translation products used in these experiments was below the level required for saturation of this in vitro import system (36). The decrease in import efficiency was also not due to the presence of import-incompetent, super-aggregated isocitrate lyase, since high molecular weight aggregates were not observed by native PAGE (Fig. 5B). Thus, we conclude that although oligomeric proteins are import competent, monomeric peroxisomal proteins are preferentially imported into plant peroxisomes in vitro.

Several studies have shown that large particles and oligomeric proteins are substrates for peroxisomal protein import (30–34). However, the import efficiency described in many of these studies is low. In one piggy-backing study, chlomaphenicol acetyltransferase constructs lacking peroxisomal targeting signals were visualized in peroxisomes 12–26 h after induction (32). In contrast, normal in vivo import occurs on the order of minutes (35, 45–47); in our in vitro system, import is detectable within 5–10 min (36). In addition, early in vivo pulse-chase experiments showed that alcohol oxidase associated with Candida boidinii peroxisomes (46) and catalase monomers associated with rat liver peroxisomes (35) before the proteins were converted to tetramers.

We cannot exclude the possibility that specific proteins or organisms (plants, mammals, yeasts, or trypanosomes) have different mechanisms of peroxisomal protein import. In another piggy-backing experiment conducted with suspension-cultured tobacco, isocitrate lyase lacking its carboxyl-terminal PTS was imported into the peroxisomal matrix when co-expressed with wild-type isocitrate lyase (34). Presumably, the mutant protein was transported to or through the membrane by heteromeric association with wild-type isocitrate lyase. The fact that isocitrate lyase may be imported as an oligomer agrees with our time course experiments. In their study, however, Lee et al. assayed peroxisomal protein import by immunofluorescence microscopy (34); quantitation of the import efficiency of the PTS-deficient construct was not possible. It is also not possible to gauge the length of time required for these constructs to gain access to the matrix.

We have demonstrated that the efficiency of isocitrate lyase import into peroxisomes is influenced both by chaperones and by the assembly state of the protein; i.e., the folding state of the peroxisomal protein is important for its import competence. Hsp90 could improve the efficiency of import by maintaining the targeted protein as a monomer, the preferred substrate for
import. Hsps might also stabilize, in a semi-folded state, a region of the protein containing the PTS, thereby exposing the signal to the cytosol and facilitating recognition by or interaction with specific peroxisomal protein receptors (48). Alternatively, Hsps could control the folding states of other proteins involved in peroxisomal protein import, such as the cytosolic PTS receptors themselves.

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