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In vitro systems in the study of peroxisomal protein import

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Abstract. Our level of understanding of peroxisome biogenesis in comparison with other cellular organelles is rudimentary, yet the fragments of information available indicate that the targeting and import of peroxisomal proteins occur by fundamentally different mechanisms. Genetic studies have identified a number of genes required for peroxisome assembly, but in most cases the functions of the gene products remain unknown. In vitro protein translocation systems have played a prominent role in unravelling the biochemistry of protein translocation into other organelles. This review considers some of the requirements for establishing a bona fide peroxisomal import assay and discusses the findings which have emerged as a result of using such experimental systems.
Key words. Peroxisomes; glyoxysomes; in vitro protein translocation assay; targeting signals; organelle biogenesis.

Introduction

Peroxisomes were discovered 30 years ago, but the past decade and a half has seen an increased appreciation of the importance of peroxisomes and peroxisomal metabolism to eukaryotic cells. This has been due to the discovery of new functions associated with peroxisomes, for example synthesis of ether lipids by mammalian peroxisomes, and the realization that a group of fatal human genetic diseases, the peroxisome biogenesis disorders (PBDs), result from a defect in protein import by peroxisomes [1]. In plants peroxisomes also play important and often different roles from their mammalian and yeast counterparts. The importance of plant peroxisomes in mobilization of storage oil reserves, synthesis of ureides and salvage of photorespiratory phosphoglycolate has been long known [2]. More recently, plant peroxisomes were discovered to be major sites of formation and detoxification of active oxygen species and have been proposed to play an important role in protecting plants from oxidative stress, which can arise as a result of environmental conditions such as pathogen attack, metal toxicity or salt stress [3].

Peroxisomes contain no DNA so are dependent on protein import for the acquisition of their protein complement. The mechanisms of peroxisomal protein import are still poorly understood. Two distinct targeting signals have been identified for matrix proteins. Peroxisome targeting signal-1 (PTS-1) is a carboxy-terminal sequence related to serine-lysine-leucine. Peroxisome targeting signal 2 (PTS-2) is an amino-terminal sequence with the consensus arginine-isoleucine or leucine-any five amino acids-histidine-leucine. The PTS-1 receptor is also known. However, what happens subsequent to the interaction of the protein-receptor complex on the peroxisome membrane is completely unknown. Recent results have demonstrated that peroxisomes, unlike mitochondria and the endoplasmic reticulum, are able to import folded proteins and even gold particles to which a peroxisome targeting signal has been attached. How they can do this whilst remaining impermeable to selected small molecules is a total mystery (see ref. 4 for a short review of recent progress in the peroxisome import field).

These results suggest that peroxisomes are quite unlike other compartments. They have a single bilayer membrane, like organelles of the endomembrane system, but are capable of importing proteins directly across that membrane like chloroplasts and mitochondria. To understand in molecular detail the events which take place as proteins traverse the peroxisome membrane should reveal new insights into the mechanisms of protein translocation across membranes and perhaps shed light on the evolutionary origins of peroxisomes.

In vitro import systems have proved immensely powerful in elucidating the events surrounding protein import into the endoplasmic reticulum (ER), mitochondria, chloroplasts and secretion from bacteria. In vitro systems epitomize the reductionist approach to solving a problem, namely to try and reduce it to simpler and simpler systems with the eventual goal of describing all the components and their functions and proving their involvement by reconstitution of protein translocation with purified components. This goal has been achieved for bacterial secretion and translocation into yeast and mammalian ER, and is within sight for the more complicated mitochondrial system where there are two separate but linked translocation systems in the mitochondrial inner and outer membranes. The logical extension of this approach is into structural studies of the components, as techniques for determining the structure of membrane proteins improve. Clearly this is not the only valid approach to solving biological problems, and it is important to corroborate in vitro results with in vivo studies for a full understanding of how protein

translocation affects the functioning of cells, and in multicellular organisms the functioning of organs and tissues. The peroxisome assembly disorders and other diseases brought about by protein mis-sorting, such as primary hyperoxaluria type 1 [5] and I-cell disease [6] illustrate the importance of accurate protein targeting at the whole organism level.

In vitro import systems for peroxisome proteins

In vitro import systems for mammalian ER [7], chloroplasts [8, 9] and mitochondria [10] were established during the second half of the 1970s. Studies on secretion in *Saccharomyces cerevisiae* and *Esherichia coli* really began with the study of mutants, and the later biochemical studies built upon this to give a satisfying picture of how these processes work. In the case of peroxisomes, early studies based on electron microscopy and subcellular fractionation were interpreted by many to invoke an origin for peroxisomes by budding from the endoplasmic reticulum, but new data which did not fit this model and re-evaluation of some of the earlier results [11] lead to the model of peroxisomes as 'autonomous' organelles which import proteins post-translationally, and can grow and divide [12]. However, it cannot be excluded that there are some proteins which are routed via the ER, although as yet there is no definitive evidence. More recently, the finding that peroxisomes could be induced in several different yeast species, including *S. cerevisiae*, has led to the isolation of many mutants defective in peroxisome biogenesis, their characterization and the cloning of the associated genes [13]. In most cases the function of these genes is understood poorly or not at all, as the biochemical analysis of peroxisomal protein import has lagged behind the genetics. The concept that peroxisomes import proteins directly across their boundary membrane established a rationale for developing in vitro import assays, but there are a number of difficulties associated with isolation of peroxisomes and measurement of import which need careful consideration when designing and carrying out in vitro import experiments.

Organelle isolation

Peroxisomes are very fragile and they are also very dense; typically sedimenting in sucrose density gradients at 1.21–1.23 $g\,cm^{-3}$, depending on the source of organelles. This is equivalent to around 1.8 M sucrose, which means that the organelles are exposed to highly hyperosmotic conditions. Thus the isolation of organelles which are sufficiently intact for import studies poses a challenge. Four factors are particularly important. These are (1) choice of the tissue; (2) breakage of the cells; (3) handling of the cell lysate and (4) isolation of the peroxisomes.

Choice of tissue. Peroxisomes and peroxisomal functions are highly inducible. Clearly an important consideration is to isolate organelles from cells which are actively importing proteins into peroxisomes. In the case of plant peroxisomes we routinely isolate glyoxysomes (a specialized type of peroxisome containing the enzymes of the glyoxylate pathway) from sunflower cotyledons 2–3 days after imbibition. The biochemistry of germination and postgerminative growth in oil seeds such as sunflower has been well established (see ref. 14 for a recent review), and at this time glyoxysomes are proliferating and approaching the maximum rate of fatty acid breakdown and its conversion to malate, which serves as a precursor for gluconeogenesis. As the cotyledons green, glyoxysomes are converted into leaf-type peroxisomes by the import of a new set of enzymes. Experiments in which thin sections are double-labelled with antibodies specific for glyoxysome enzymes and leaf peroxisome enzymes have shown that the leaf peroxisome enzymes are imported by pre-existing organelles. During senescence a reverse transition takes place, and fatty acid oxidation and glyoxylate pathway enzymes are reimported to permit breakdown of membrane lipids and the export of this carbon to the rest of the plant from the cotyledon before it is shed. Thus cotyledon peroxisomes probably remain import-competent throughout their life.

It is not clear if this is the case with peroxisomes from other sources. Evidence has been presented, based on subcellular fractionation and electron microscopy of rat liver, that peroxisomes are very heterogeneous and proteins are preferentially imported into smaller, less dense organelles, leading to the suggestion that 'mature' peroxisomes may not be import-competent [15, 16]. Another important consideration is the possibility that under some conditions peroxisomes may form an interconnected reticulum. It is not known whether fragments of this reticulum will reseal to form import-competent vesicles, as occurs with microsomes. As at present there are no definitive answers to these questions; empirical approaches to the choice of tissue are unavoidable.

Cell breakage and handling of the lysate. Cell breakage depends on the source of the tissue and reflects a compromise between efficiency of cell breakage and possible damage to peroxisomes during homogenization. For isolating glyoxysomes from germinating oil seed cotyledons, which possess only thin primary cell walls, we prefer to use manual chopping with a food chopper or razor blades followed by filtration of the homogenate and a 1000 *g* centrifugation to remove cellular debris before loading the resulting postnuclear supernatant directly onto a gradient [17]. This reduces handling, as pelleting and resuspension of plant glyoxysomes has a marked detrimental effect on their subsequent import competence. The homogenization conditions are also important and should be established

by comparison of different conditions. In our hands the most significant factor in isolating intact plant peroxisomes is the pH. As has been reported for yeast peroxisomes [18], isolation of plant peroxisomes at pH 6 gives superior yields and intactness; however, protocols for isolating rat liver peroxisomes use buffers at pH 7.4–7.6 and produce satisfactory results [19, 20].

Estimating the intactness of peroxisomes also represents a problem. Conventionally, latency is taken as a measure of the sequestration of an enzyme within a membrane, provided that at least one of the substrates or products is membrane impermeable. Unfortunately, little is known about the transport of metabolites across peroxisome membranes, largely due to the difficulty of isolating intact organelles. The fact that catalase in peroxisomes always displays a high degree of latency (e.g. ref. 19) despite the substrate, H_2O_2, and the products, H_2O and O_2, being membrane permeable, indicates that latency is perhaps not the best measurement of membrane integrity. In our experience we see little or no latency of isocitrate lyase in glyoxysome preparations, whereas malate synthase activity is stimulated severalfold by procedures designed to rupture the organelles [17]. In the case of leaf peroxisomes latency of hydroxypyruvate reductase was observed in osmotically shocked organelles which were demonstrated by electron microscopy to have lost an intact boundary membrane [21]. Latency in peroxisomes can therefore be the result of association of many of the enzymes into very stable multienzyme complexes which serve to protect cells from the toxic intermediates generated by peroxisomal metabolic pathways, and may therefore be misleading with regard to membrane integrity. At present there is no satisfactory solution to this problem, as electron microscopy is clearly not a practical alternative for routine monitoring of organelle intactness.

Isolation of peroxisomes from cell lysates. In preparing peroxisomes for in vitro protein import assays, the primary objective is the isolation of import-competent organelles. Issues of yield and purity are of secondary importance, as long as sufficient material can be prepared to carry out the experiment and contaminants can be quantified and their effects on the import assay controlled. In principle, crude organelle fractions can be used for import assays and the organelles separated post-import [22–24] thus circumventing the need to expose the peroxisomes to hyperosmotic conditions prior to carrying out the import assay. However in such experiments careful controls to prove the specificity of import are especially important (see later). In our hands this approach is not successful with sunflower glyoxysomes due to high levels of protease activity present in crude organelle fractions, even in the presence of common protease inhibitors. Thus most successful in vitro protein import protocols further purify the peroxisomes by density gradient centrifugation. Most procedures use Nycodenz as the gradient medium, as solutions of high density suitable for isolating peroxisomes have relatively low osmolality [25]. Imanaka et al. [19] attributed the use of Nycodenz gradients in place of sucrose as the most important factor in the improvement of their rat liver peroxisome import assay.

The import assay

Unlike the majority of mitochondrial-, chloroplast- and ER-targeted proteins, most peroxisomal proteins do not undergo proteolytic processing, or other well defined post-translocational modifications such as glycosylation, associated with import. This means that determining whether a protein is imported or not depends upon a protease protection assay (fig. 1). For matrix proteins, conditions must be established where there is complete protection from externally added protease in intact organelles, but complete degradation of the full-length protein when the organelles are solubilized, for example by detergents or sonication. For membrane proteins, it is important to establish that the protein is correctly inserted in the membrane. Some membrane proteins may have cytosolically exposed domains which will be accessible to protease, whereas others may not. It is therefore important to compare patterns of digestion of the protein in the native membrane to the material imported in vitro (e.g. refs 26, 27). Other criteria which may help to establish the correct insertion of membrane proteins are whether or not they are sensitive to extraction by alkaline sodium carbonate and/or Triton-X-114 phase partitioning. As different proteins differ in their sensitivity to proteases, it is necessary to check for each protein that digestion conditions are sufficient to degrade that protein in the presence of detergent-lysed organelles and that the peroxisomes themselves are not damaged by the protease treatment. This is usually done by comparing stained protein profiles of treated vs untreated peroxisomes [28], by immunoblotting for a matrix marker [17] or by monitoring the effect of protease treatment on latency of a peroxisomal enzyme [19, 27], although the limitations of latency measurements as an estimate of peroxisome intactness should be borne in mind. At high protease concentrations peroxisome integrity is compromised, so the import of proteins which require more than this 'threshold' of protease for degradation cannot be studied [19, 24]. This is one of the major disadvantages of protease protection as a criterion for protein import.

Clearly it is important to establish that the protein under investigation is imported into peroxisomes and not other organelles. Specificity can be demonstrated by replacing the peroxisomes with another cellular organelle; mitochondria are often used [17, 19, 27], but in principle any other membrane-bound compartment can be used. When studying a new protein it is also important to

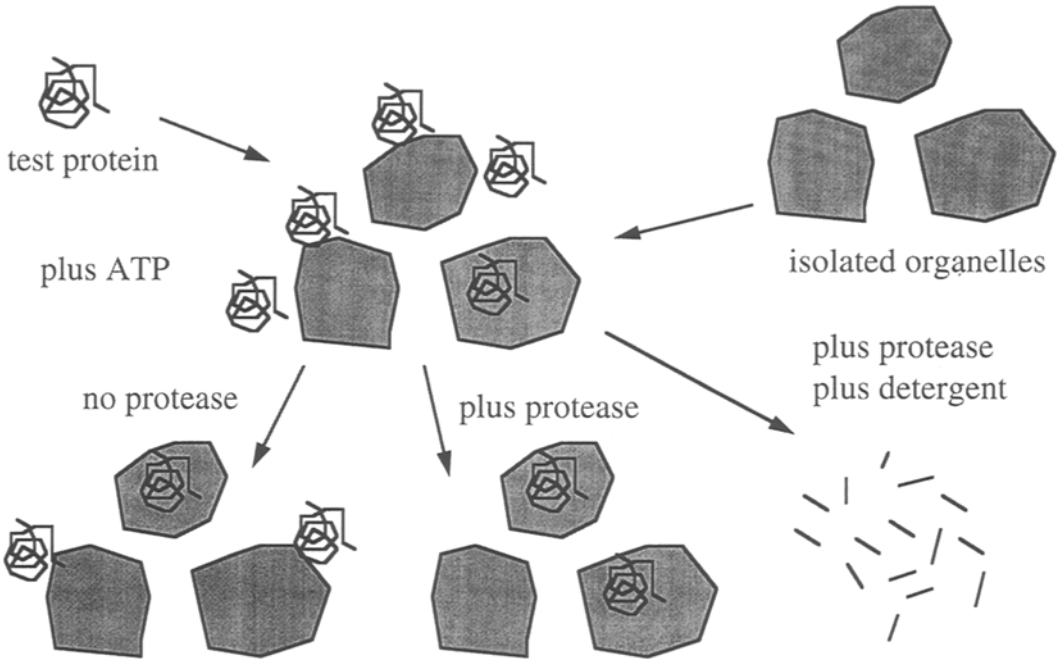

Figure 1. Cartoon representation of an in vitro protein translocation assay. The test protein is normally produced in radiolabelled form by in vitro transcription and translation of a cloned cDNA sequence. This is then mixed with isolated peroxisomes in the presence of ATP and incubated at the desired temperature for the appropriate length of time. Three duplicate assays are set up or one assay is split three ways at the end of the incubation period. One of the assays is treated with protease using predetermined conditions. A second aliquot is subjected to a control incubation without protease. The third aliquot is centrifuged to reisolate the organelles, which are then lysed and treated with protease under the same conditions used for the first aliquot. At the end of the protease digestion period, the protease is inhibited, and the organelles from the first two incubations reisolated by centrifugation through a sucrose cushion to remove unbound material. Pellet and supernatant fractions can then be analysed by SDS-polycrylamide gel electrophoresis and fluorography.

include controls where organelles are omitted altogether to ensure that protease-resistant aggregates are not being formed. When setting up a peroxisome protein in vitro import system, it is also important to check that nonperoxisomal proteins do not show protease resistance when incubated with peroxisomes. These controls were often neglected in earlier studies [22, 29, 30].

The transport of proteins across membranes is almost always dependent upon ATP hydrolysis [31]. In vitro import experiments established that peroxisome protein import was also ATP-dependent [19], and this was subsequently confirmed by studies with semipermeabilized cells [32, 33]. This finding also provides an additional very useful control. If ATP is depleted in an import assay, no protease-protected species should be seen. As there are many spurious ways in which apparent protease protection can be generated (e.g. aggregation, insufficient protease), we feel it is important to include a minus ATP control in every experiment. However, this is not applicable to membrane proteins, the insertion of which appears to be ATP-independent (see later).

What in vitro systems have taught us

Targeting

The main use of peroxisome in vitro import systems so far has been in the study of targeting sequences. Rat liver acyl coenzyme A (CoA) oxidase (AOX) is the best characterized protein in terms of its targeting signal. Miyazawa et al. [24] carried out a deletion analysis of the rat AOX gene and tested the mutants using an in vitro import assay. They found that the extreme carboxy-terminus of the protein was essential for import into peroxisomes and that regions immediately upstream of the carboxy-terminus contributed to efficiency. The last 199 amino acids when attached to chloramphenicol acetyl transferase or dihydrofolate reductase could target these passenger proteins to peroxisomes. Due to technical difficulties with protease-resistant fusion proteins it was not possible to demonstrate whether the last five amino acids were sufficient as well as necessary for targeting either passenger protein. Subsequently, it was shown that the last three or five amino acids of AOX when attached at the carboxy-terminus of import negative carboxy-terminal deletion mutants of catalase or AOX could restore import [20]. The last three amino acids of AOX are serine-lysine-leucine, and deletion or nonconservative substitution of these residues inhibited import of the protein. Import of acyl CoA oxidase was inhibited by a synthetic peptide comprising the last 10 amino acids of the protein, but not by peptides corresponding to nonfunctional derivatives of AOX, although the concentrations used in these experiments (up to 100 μM) were very high. Amidation of the

peptide corresponding to the wild-type AOX sequence abolished its ability to act as a competitor, demonstrating that a free α-carboxyl group is important in recognition of the targeting sequence.

Similar results were also reported for urate oxidase from rat liver, which ends with the tripeptide sequence serine-arginine-leucine [34]. Mutation of this sequence to serine-lysine-leucine supported import of the protein, but serine-glutamic acid-leucine did not. The acyl CoA oxidase-derived peptide could competitively inhibit import of urate oxidase, showing that it uses the same import pathway as AOX. These results are in complete agreement with independent in vivo studies on targeting of firefly luciferase to mammalian peroxisomes [35].

Although *Candida tropicalis* and rat liver AOX are quite similar, the *Candida* AOX does not end with the tripeptide sequence serine-lysine-leucine. When deletion mutants were tested for their ability to be imported into *C. tropicalis* peroxisomes in an in vitro import assay, two separate regions, one within the first 112 amino acids and one within the central portion of the protein were identified which were required for import activity [36]. Residues 1–118 and residues 309–427 when fused at the amino-terminus of dihydrofolate reductase could direct this passenger protein into peroxisomes, although the efficiency was low, 14 and 5% respectively. However, native dihydrofolate reductase was not imported. The nature of the targeting information in AOX has not been further elucidated, but genetic studies indicate that *S. cerevisiae* AOX is also not imported by the PTS-1 pathway [37].

The ability of isocitrate lyase (ICL) from castor bean to be imported into isolated sunflower glyoxysomes was examined and found not to be dependent upon a putative PTS-1 sequence located at the carboxy-terminus [17]. This was subsequently confirmed in vivo [38], although *Brassica napus* isocitrate lyase is dependent on a carboxy-terminal PTS for import into peroxisomes in transgenic *Arabidopsis* plants [39]. The results with AOX and ICL suggest that the same enzyme from different species can be imported by different routes.

Import mechanisms

Energy. In vitro import assays provided the first demonstration that ATP hydrolysis was required for peroxisome protein import, but not for binding [19]. No evidence for the requirement of a proton motive force was detected. These results were subsequently confirmed by studies using permeabilized cells [32, 33] and in vitro import assays with other matrix proteins [17, 40]. In contrast, insertion of the rat liver peroxisome membrane proteins PMP22 and PMP70 did not require ATP [27, 28], consistent with genetic data which indicate that membrane proteins are inserted by different mechanism(s) to matrix proteins.

Temperature. In most cases examined to date binding to the peroxisome membrane can occur at low temperature, but import or membrane insertion cannot. PMP70 appears to be an exception in that binding is also inhibited at low temperature [28].

These results suggest a two-step mechanism for protein import into peroxisomes (fig. 2).

Cytosol. In permeabilized mammalian cell systems cytosol is absolutely required for import of human serum albumin derivatized with peptides bearing the peroxisome targeting signal serine-lysine-leucine (HSA-SKL; [32]) but in in vitro systems there is no clear-cut requirement; the import of catalase [23] and insertion of rat liver PMP70 into peroxisome membranes [28] was stimulated by the addition of cytosol, but acyl CoA oxidase was not markedly affected [23]. A possible candidate for a cytosolic factor is the receptor for the PTS-1 targeting signal. There is controversy regarding the localization of this protein. In the yeast *Pichia pastoris* the receptor (encoded by the *PAS8* gene), is predominantly associated with the peroxisome membrane [41], but in mammalian cells it may be mostly cytosolic [42]. Current models propose that the receptor may cycle between the cytosol and peroxisomal membranes. Other proteins which have been postulated to be involved in protein translocation into peroxisomes are the hsp70 group of chaperone proteins [43]. Their role is thought to be maintaining proteins in a translocation-competent form. How this fits with recent data showing that peroxisomes can import folded substrates is unclear [44].

In in vitro import experiments it is possible that proteins from the translation system (wheat germ or reticulocyte lyase) can meet any requirement for cytosolic factors, or that sufficient cytosolic proteins are associated with the organelles to permit the low rates of transport seen in vitro. Alternatively, the requirement for cytosolic components may be protein-dependent as is the case for mitochondrial precursor proteins.

Inhibitors. The thiol-modifying reagent *N*-ethylmaleimide (NEM) inhibits the uptake of HSA-SKL by peroxisomes in permeabilized cells. Treatment of the cytosol with NEM had no effect, indicating that the NEM-sensitive component is associated with the organelles [32]. Import of acyl CoA oxidase in vitro was inhibited by treatment of peroxisomes with NEM [28], but insertion of the membrane proteins PMP70 [28] and PMP22 [27] was not affected by NEM treatment of either peroxisomes or cytosol. Dithiothreitol did not enhance import of acyl CoA oxidase into *C. tropicalis* peroxisomes [36] or the import of isocitrate lyase into sunflower glyoxysomes (M. Pool and A. Baker, unpublished observations).

The polysulphonate compounds suramin (a drug used in the treatment of trypanosomiasis), Cibacron Blue F3GA and trypan blue used at concentrations of 10 μM all inhibited the import of rat acyl CoA oxidase into rat

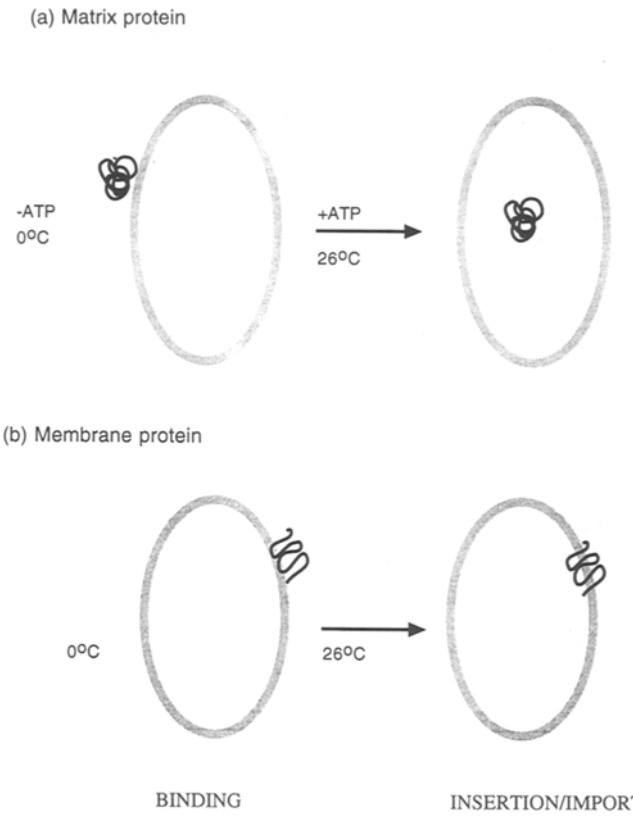

Figure 2. Illustration of two-step model of import for (a) peroxisomal matrix and (b) membrane proteins. See text for details.

liver peroxisomes, but other sulphonate compounds did not inhibit at up to 60 μM. Incubation of peroxisomes with 10 μM suramin did not decrease catalase latency or affect ATP hydrolysis in the import assay. Treatment of the translation products with suramin had no effect on import efficiency, suggesting that the peroxisomes are the site of inhibition [45]. The mechanism of action of suramin remains unknown.

Conclusions and future prospects

The use of in vitro import assays to study protein translocation into peroxisomes has provided some important insights into targeting and import mechanisms. The major limitation to date has been the relatively low efficiencies of protein import achieved. While efficiencies of up to 30% have been reported [19, 40] 5–10% or even less is more common [20, 24, 34, 36]. This may indicate that the existing systems are not optimal, but is almost certainly in part due to the fragility of the peroxisomes. Protease protection may underestimate the amount of protein imported because of damage to the peroxisomes through handling during import and subsequent procedures. This is a particular problem when attempting to carry out a two-step import reaction, for example when proteins are bound to the surface of the peroxisomes, the peroxisomes reisolated and then the conditions altered to allow import. When PMP22 was imported in such a two-step assay, the level of protease protection was half that seen in a single-step import assay incubation [27]. Nevertheless, this type of experiment is crucial in order to be able to investigate the requirements for different phases of the import reaction.

The problems associated with peroxisome fragility may be something that experimentalists have to live with. However, if the amount of imported protein can be increased by improving the routine efficiency of the import assay or by scaling up, the losses may be bearable. We have recently begun to use a recombinant purified protein as a substrate for the import assay and estimate that up to 500 ng of protein can be imported per 200–300 μg of glyoxysomes (M. Pool and A. Baker, unpublished results). This is easily detectable by Western blotting and opens up the possibility to carry out experimental manipulations which are not possible with the tiny quantities of protein produced by in vitro transcription and translation.

Peroxisome biogenesis is at an exciting stage. There is still much to be learned, but already it is apparent that

these organelles are fundamentally different in the way that they import proteins. Genetic approaches have identified many genes involved in this process; the challenge now is to integrate the genetics with biochemistry to find functions for all these gene products. As has been the case with the study of protein translocation into other organelles, in vitro systems are likely to play an important part in this process.

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