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Import of Stably Folded Proteins into Peroxisomes

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By virtue of their synthesis in the cytoplasm, proteins destined for import into peroxisomes are obliged to traverse the single membrane of this organelle. Because the targeting signal for most peroxisomal matrix proteins is a carboxy-terminal tripeptide sequence (SKL or its variants), these proteins must remain import competent until their translation is complete. We sought to determine whether stably folded proteins were substrates for peroxisomal import. Prefolded proteins stabilized with disulfide bonds and chemical cross-linkers were shown to be substrates for peroxisomal import, as were mature folded and disulfide-bonded IgG molecules containing the peroxisomal targeting signal. In addition, colloidal gold particles conjugated to proteins bearing the peroxisomal targeting signal were translocated into the peroxisomal matrix. These results support the concept that proteins may fold in the mammalian cytosol, before their import into the peroxisome, and that protein unfolding is not a prerequisite for peroxisomal import.

INTRODUCTION

Peroxisomes are similar to mitochondria, chloroplasts, and the nucleus in that most of their proteins are synthesized on cytoplasmic polysomes and imported into the organelle post-translationally (reviewed by Subramani, 1993). Although the conformational requirements for translocation across other cellular membranes are known in some detail, they are presently unknown for the peroxisomal membrane. The cytosol of eukaryotic cells is endowed with chaperone molecules capable of facilitating the productive folding and oligomerization of peroxisomal proteins. The peroxisomal protein luciferase can be folded properly in the presence of cytosolic chaperones (Frydman *et al.*, 1992). In cells derived from patients with Zellweger syndrome (deficient in the import of catalase and other SKL proteins), monomers of catalase appear to be folded and assembled into catalytically active tetramers (Wanders *et al.*, 1984). Similarly, in yeast mutants lacking peroxisomes, octameric alcohol oxidase and other peroxisomal oxidases are assembled in the cytosol (Cregg *et al.*, 1990; Sulter *et al.*, 1990; van der Klei *et al.*, 1991). In addition, peroxisomal thiolase appears to dimerize in yeast cytosol before import into peroxisomes (Glover *et al.*, 1994).

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The mitochondrial import machinery accommodates hybrid proteins or even DNA (Vestweber and Schatz, 1989). However a substantial body of evidence suggests that proteins must be at least partially unfolded to be translocated across the mitochondrial membranes (Pfanner and Neupert, 1990; Hannavy *et al.*, 1993). Proteins initially assuming a folded configuration in the cytosol can be disassembled and unfolded during import (Skerjanc *et al.*, 1990). Indeed, a cytosolic protein has been found to depolymerize and unfold mitochondrial precursor proteins, stimulating import (Hachiya *et al.*, 1993). The rate of translocation into mitochondria has been shown to increase if the proteins are first denatured by urea (Eilers *et al.*, 1988) or destabilized by point mutations (Vestweber and Schatz, 1988a). The tightly folded and disulfide-bonded bovine pancreatic trypsin inhibitor blocked the transport of an artificial mitochondrial precursor protein into the mitochondrion (Vestweber and Schatz, 1988b). Large multisubunit complexes such as IgG molecules are also import incompetent in mitochondria (Schleyer and Neupert, 1985).

Protein unfolding is not required for nuclear transport. To determine the dimensions of a protein that could traverse the nuclear pore, Feldherr and colleagues (Feldherr *et al.*, 1984; Dworetzky and Feldherr, 1988) microinjected colloidal gold particles coated with nucleoplasmin or RNA into *Xenopus* oocytes.

Their results demonstrated that the nuclear pore could permit the entry of molecules as large as 23 nm in diameter, as would be required for passage of large ribosomal subunits.

Assays based on microinjection (Walton *et al.*, 1992a–1994; Soto *et al.*, 1993) and cell permeabilization (Wendland and Subramani, 1993) have demonstrated that prefolded proteins, such as luciferase or human serum albumin (HSA) coupled to a 12-mer peptide (CRYHLKPLQSKL) ending in SKL (HSA-SKL) are imported into mammalian peroxisomes. HSA contains 17 disulfide bonds. Although the reductive environment of the cytosol is predicted to prevent disulfide formation (Hwang *et al.*, 1992), it is not known whether a protein that contains disulfide-bonded cysteines could be reduced. In these studies, the state of folding of these proteins at the time of translocation across the peroxisomal membrane was unknown. In the present study, we sought to determine whether stably folded proteins were substrates for peroxisomal import.

MATERIALS AND METHODS

Reagents

Biotinylated human serum albumin (bHSA) was prepared as described previously (Walton *et al.*, 1994), except that the second step, the addition of the peroxisomal targeting signal, was omitted. bHSA-s-s-SKL was prepared as described previously (Walton *et al.*, 1992a) except that the sulfo-MBS was replaced with the disulfide-containing cross-linker SPDP (Pierce, Rockville IL). Nonspecific rat IgG (Sigma Chemical, St. Louis, MO) containing the peroxisomal targeting signal serine-lysine-leucine (IgG-SKL) was prepared as described previously (Walton *et al.*, 1992a). bHSA-SKL with various degrees of intramolecular cross-linking was prepared as described previously (Walton *et al.*, 1994), except that the reagents were diluted 10-fold to minimize intermolecular cross-linking, and DSP (Pierce) was added to the first incubation at molar concentrations of between 0–20 times that of the HSA.

Rabbit anti-catalase antibodies were obtained from Calbiochem (La Jolla, CA). Mouse anti-β-tubulin antibodies were a generous gift from Dr. J. Feramisco (University of California-San Diego). Streptavidin-conjugated Texas Red was purchased from Amersham (Oakville, ON). Species-specific fluorescently labeled secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Other reagents were obtained from the standard sources.

Cell Culture

The human fibroblast cell line, Hs68, was obtained from American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% FCS (Life Technologies, Gaithersburg, MD). For microinjection followed by immunofluorescence, cells were plated on acid-washed glass coverslips.

Microinjection and Recovery of bHSA

Cells were microinjected using glass capillary needles as previously described (Walton *et al.*, 1992a). bHSA was microinjected at a concentration of 5 mg/ml in a buffer of 20 mM potassium phosphate (pH 7.4), 100 mM KCl. With an average injection vol of 5×10^{-14} liters, approximately 2×10^6 molecules of bHSA were injected per cell. Approximately 200 cells were microinjected, about 0.7 fmol of bHSA in total. After microinjection, the cells were incubated for 24 h at 37°C. After the incubation, the cells were washed with phosphate buffered-saline (PBS), scraped into Laemmli sample buffer plus or minus 50 mM dithiothreitol (DTT), the proteins separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose paper. The Western blot was probed with ^{35}S-streptavidin (Amersham), and visualized with a Molecular Dynamics Phosphorimager (Sunnyvale, CA). Control experiments indicated that oxidized bHSA was recognized by streptavidin approximately 50 times less efficiently than reduced bHSA; we assume that this was due to steric hindrance in the case of the more fully folded protein.

Microinjection and Immunofluorescence

Cells were microinjected with approximately 10^5 molecules of bHSA-s-s-SKL, bHSA-SKL, or IgG-SKL and incubated for 16 h at 37°C. Cells were then washed with PBS and permeabilized with digitonin (25 μg/ml) for 10 min. After the digitonin treatment the cells were fixed, permeabilized, and immunostained. This employed rabbit anti-catalase antibodies in the first step, and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibodies plus streptavidin-conjugated Texas Red or Texas Red-conjugated donkey anti-rat antibodies in the second step. To confirm that the microinjected proteins were in a membrane-enclosed compartment, the Triton X-100 step was omitted from control experiments, as indicated in the figure legends. Fluorescence microscopy was performed with a Zeiss Axiophot Photomicroscope or a Zeiss LSM 420 confocal microscope using a 63× (1.3NA) lens (Thornwood, NY).

Preparation of Colloidal Gold and Electron Microscopy

Colloidal gold particles were prepared by the reduction of gold chloride (Slot and Geuze, 1985), and were coated with 1.5 mg bHSA-SKL and subsequently with 100 mg HSA as an additional stabilizer. Colloidal gold/protein complexes were isolated and separated from unbound proteins by centrifugation, and the buffer changed to injection buffer by Centricon filtration. For electron microscopic examination, Hs68 cells were grown in 35-mm plastic tissue culture dishes. To facilitate the identification of microinjected cells, circles of approximately 2 mm were drawn on the bottom of the dishes and all cells within the circles were microinjected. After microinjection of the gold/bHSA-SKL complex, the cells were incubated for 16 h at 37°C. Cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated with graded alcohols, and embedded in Epon. Blocks containing the microinjected cells were sawn out of the Epon pucks, sectioned, and mounted on grids, and stained with uranyl acetate and lead citrate before observation. For immunoelectron microscopy the osmium tetroxide step was omitted. These sections were mounted on gold grids, etched with 10% H_2O_2, and immunostained with antibodies directed against catalase (1:5 dilution in PBS plus 2% BSA, 2% casein, 0.5% ovalbumin). The secondary reagents were 10 nm gold-labeled goat anti-rabbit antibodies (Sigma) (1:20 dilution). Electron microscopy was performed with a Phillips 400T electron microscope at 80 KeV.

RESULTS

HSA Remains Disulfide Bonded after Microinjection into Mammalian Cells

To determine whether bHSA could be reduced in the cytosol, facilitating its unfolding, this molecule was recovered after microinjection and assayed for its state of folding. We made use of the observation that unfolded HSA migrates more slowly (Figure 1a, lane 1)

during SDS-PAGE than does the disulfide-bonded form (Figure 1a, lane 2). Incubation of HSA in cytosol of living cells for 24 h did not result in the reduction of the disulfide bonds (Figure 1a, lane 3). The reisolated bHSA migrated with an apparent molecular mass of 51 kDa, the same molecular mass as the nonreduced form. A control experiment (Figure 1a, lane 4) showed that the bHSA recovered from injected cells could be reduced artificially by the addition of dithiothreitol (DTT) before SDS-PAGE. As a further test of the stability of disulfide bonds, bHSA-s-s-SKL prepared with a disulfide bond in the cross-linker was microinjected into Hs68 cells and incubated for 18 h at 37°C. This molecule was imported into peroxisomes (Figure 1b), indicating that the disulfide bond was stable in the cytosol. With stable disulfide bonds, the microinjected albumin molecules are expected to have retained much of their tertiary structure during the import assays.

Intramolecular Cross-linking Has No Effect on the Import of bHSA-SKL

The binding of methotrexate to a mitochondrial DHFR hybrid protein stabilized the tertiary structure of the enzyme and prohibited its import into the mitochondrion (Eilers and Schatz, 1986). Ligand-induced inhibition of transport has also been demonstrated for proteins destined for the chloroplast (della Cioppa and Kishore, 1988), indicating that these organelles require some degree of protein unfolding before import. The effects of stabilization of the tertiary structure, by chemically cross-linking a protein, on its ability to be imported into the peroxisome were studied using the hybrid protein bHSA-SKL. Hybrid proteins, prepared with increasing amounts of cross-linker, displayed faster migration rates in SDS gels as their ability to unfold was increasingly restricted (Figure 2A). When the most cross-linked of these hybrid proteins (Figure 2A, lane 5) was microinjected into Hs68 cells, it was imported into peroxisomes in a manner that was indistinguishable from native bHSA-SKL (Figure 2B). The cross-linked bHSA-SKL molecules were imported into peroxisomes, as indicated by their colocalization with catalase, a bona fide peroxisomal marker (Figure 2B, panels a and b). In this injected sample, less than 5% of the material would have corresponded to uncross-linked bHSA-SKL. In our experience with this substrate, microinjection of even 20% of the substrate is too little to yield a signal by immunofluorescence. Thus, the peroxisomal localization of the cross-linked bHSA-SKL (Figure 2B, panel a) cannot be accounted for by the presence of uncross-linked material in the sample. Indeed, all of the cross-linked samples were import competent (Walton, unpublished observations). As a control, confirming that the bHSA-SKL was indeed within the peroxisomal membrane, duplicate experiments were conducted omitting the Triton X-100 permeabilization step. This pro-

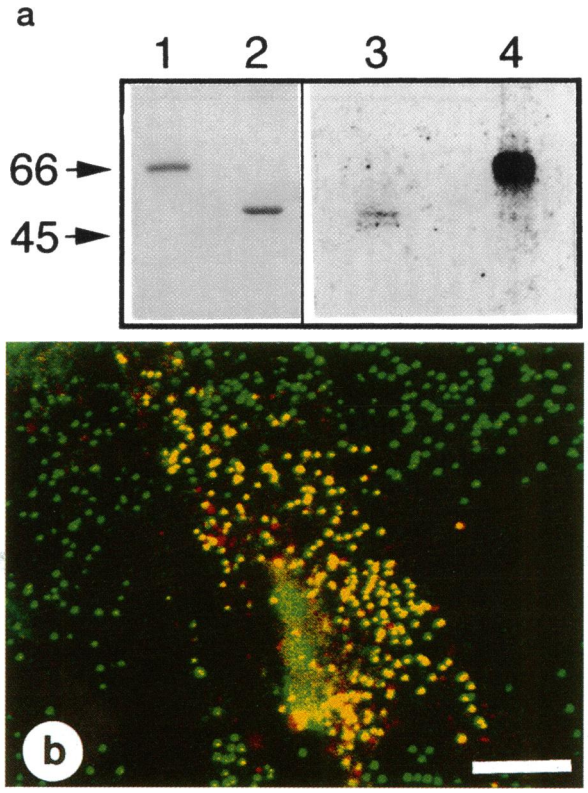

Figure 1. Disulfide bonds are not reduced in the cytosol of Hs68 cells. (a) The electrophoretic mobility of reduced (lane 1) and nonreduced (lane 2) bHSA. Each lane contained 6 μg of bHSA that was stained with Coomasie blue. The reduction of the disulfide bonds allowed greater unfolding of the molecule, and thus a slower migration through the gel. Numbers at left represent the apparent molecular mass of reduced protein standards (in kilodaltons). Lane 3, electrophoretic mobility of bHSA microinjected into Hs68 cells, and reisolated from the cytosol in Laemmli sample buffer minus DTT. After 24 h in the cytosol of Hs68 cells, the bHSA remained oxidized, and as such ran at the faster molecular weight in the SDS-PAGE gel. Lane 4, bHSA that had been microinjected into the Hs68 cells, and was isolated in Laemmli sample buffer that contained DTT. The bHSA in this sample had been reduced by the DTT in the sample buffer, and thus ran at the higher molecular weight. (b) Peroxisomal import of microinjected bHSA-s-s-SKL. This hybrid protein was constructed with a disulfide bond between the HSA molecule and the peroxisomal targeting signal. Hs68 cells were microinjected with bHSA-s-s-SKL and incubated for 16 h at 37°C. Cells were subsequently washed with PBS and permeabilized with digitonin (25 μg/ml) in PBS for 10 min. After the digitonin treatment the cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and immunostained for bHSA-s-s-SKL and endogenous catalase. Staining consisted of rabbit anti-catalase as the primary reagent and FITC-conjugated donkey anti-rabbit antibodies and streptavidin-conjugated Texas Red as secondary reagents. Identical confocal microscopic sections were scanned for both fluorochromes. Figure shows the false-color overlay indicating vesicles that contain catalase (green), bHSA-s-s-SKL (red), or both (yellow). Import was into endogenous peroxisomes as confirmed by colocalization with catalase. Bar, 5 μm.

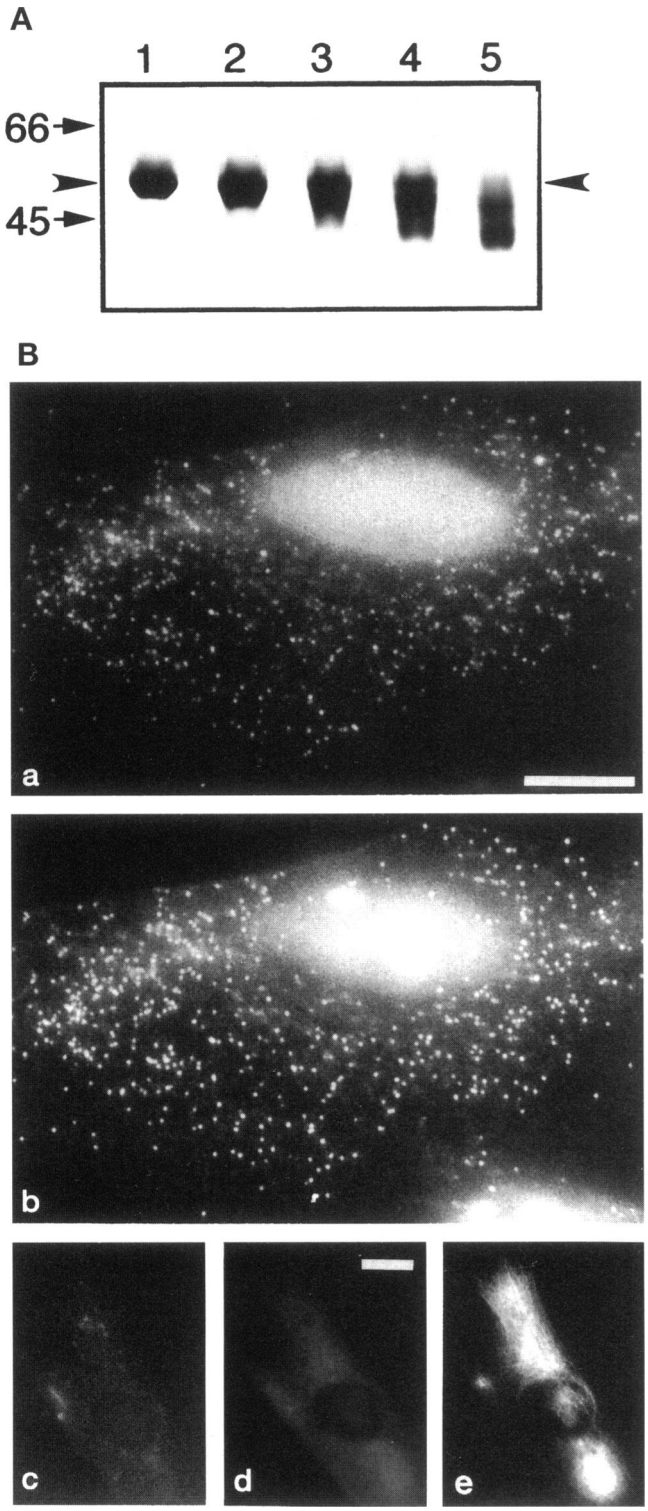

Figure 2. Effects of chemically cross-linking bHSA-SKL on its electrophoretic mobility (A) and on its import into peroxisomes (B). (A) In nonreducing SDS-PAGE gels, the native HSA-SKL molecule migrated with an apparent molecular mass of 51 kDa (lane 1). The addition of cross-linker resulted in the appearance of two faster migrating molecular species. Initially a faster migrating molecule with an apparent molecular mass of 47 kDa was observed when the HSA was incubated with DSP at between 2–10 times the molar concentrations (lanes 2–4). At a 10 to 20-fold excess of cross-linker a still faster migrating form was observed with an apparent molecular mass of 42 kDa (lanes 4 and 5). The original, 51-kDa form (arrowheads) appears to have been completely modified by the incubation of HSA with a 20-fold excess of cross-linker (lane 5). These faster migrating forms represent cross-linked structures with an increasingly restricted ability to unfold in SDS-PAGE sample buffer. That their appearance seems to occur in steps, and no further forms appear at higher levels of DSP, suggests that there might be two pairs of lysine residues within the 1.2-nm range of the cross-linker. Numbers at left represent the apparent molecular mass of protein bands (in kilodaltons). (B) Peroxisomal import of microinjected cross-linked bHSA-SKL (sample from lane 5). Hs68 cell were incubated and immunostained as described in Figure 1b. Import of the cross-linked bHSA-SKL (a) was into endogenous peroxisomes as confirmed by costaining for catalase (b). As a control to confirm that the bHSA-SKL was within the peroxisomal matrix, omission of the Triton X-100 permeabilization step from the staining protocol resulted in the loss of the punctate staining for bHSA-SKL (c) and catalase (d), but not for β-tubulin (e). Bar, 5 μm.

tocol, based upon the selective permeabilization of the cholesterol-rich plasma membrane leaving the peroxisomal membrane intact (Wanders *et al.*, 1984), has been used previously to demonstrate that a membrane exists between the cytosol and the imported peroxisomal proteins (Walton *et al.*, 1992a,b; Wendland and Subramani, 1993). Failure to permeabilize the organellar membranes resulted in the loss of the punctate staining for bHSA-SKL (Figure 2B, panel c) and catalase (Figure 2B, panel d), although antibodies were able to detect the microtubules (Figure 2B, panel e).

IgG Coupled to a PTS-1 Peptide Is Imported into Peroxisomes

Recent results have demonstrated that thiolase dimers and chloramphenicol acetyltransferase trimers are imported into peroxisomes from *Saccharomyces cerevisiae* (Glover *et al.*, 1994; McNew *et al.*, 1994). We sought to determine whether mammalian cells were capable of the import of multimeric protein complexes. Large, multisubunit complexes such as IgG molecules, tagged with mitochondrial targeting signals are not imported into mitochondria (Schleyer and Neupert, 1985). IgG molecules, lacking a peroxisomal targeting signal, have been commonly co-injected into Hs68 cells as a marker to identify microinjected cells. They remain cytosolic and not peroxisomal (Walton *et al.*, 1992a–1994). In contrast, when rat IgG cross-linked with the peptide (CRYHLKPLQSKL) containing the SKL peroxisomal targeting signal (PTS-1) was microinjected into human (Hs68) fibroblasts, the hybrid IgG-SKL molecules were imported into peroxisomes, as indicated by their colocalization with catalase (Figure 3, a and b). Control experiments demonstrated that failure to permeabilize the organellar membranes

resulted in the loss of the punctate staining for IgG-SKL (Figure 3c) and catalase (Figure 3d), although antibodies were able to detect the microtubules (Figure 3e).

Import of Colloidal Gold Particles Bearing the Peroxisomal Targeting Signal into the Matrix of the Mammalian Peroxisome

To determine whether the peroxisomal import machinery could import a large, nondeformable complex, colloidal gold particles coated with bHSA-SKL were microinjected into Hs68 cells. After the standard incubation time of 16 h, the microinjected cells were processed for electron microscopy. Electron micrographs clearly show colloidal gold particles within the limits of single-membrane bound structures (Figure 4, a–c). These structures were confirmed to be peroxisomes by the presence of catalase (Figure 4d). The specificity of this import was confirmed by the observation that few, if any, gold particles were found in association with other subcellular compartments. As an additional control, co-injection of a synthetic peptide bearing a peroxisomal targeting signal that has previously be shown to inhibit peroxisomal import (Walton *et al.*, 1992a, b; Wendland and Subramani, 1993), resulted in a reduction by approximately two-thirds in the number of peroxisomal profiles containing gold particles and a reduction in the average number of gold particles observed per peroxisomal profile by approximately 85% (Figure 5). These experiments demonstrate that the gold particles (and hence the bHSA-SKL) gain entry into the peroxisomal matrix in a PTS1-dependent manner. Electron microscopic examination of the imported gold particles indicated that their diameters ranged from 4–9 nm. Our results confirm that multimeric complexes can be imported into peroxisomes, even if one of the constituents is a metal particle. Furthermore, the dimensions of a particle that can traverse the peroxisomal membrane is at least 9 nm, the largest gold particle observed within the peroxisomal matrix.

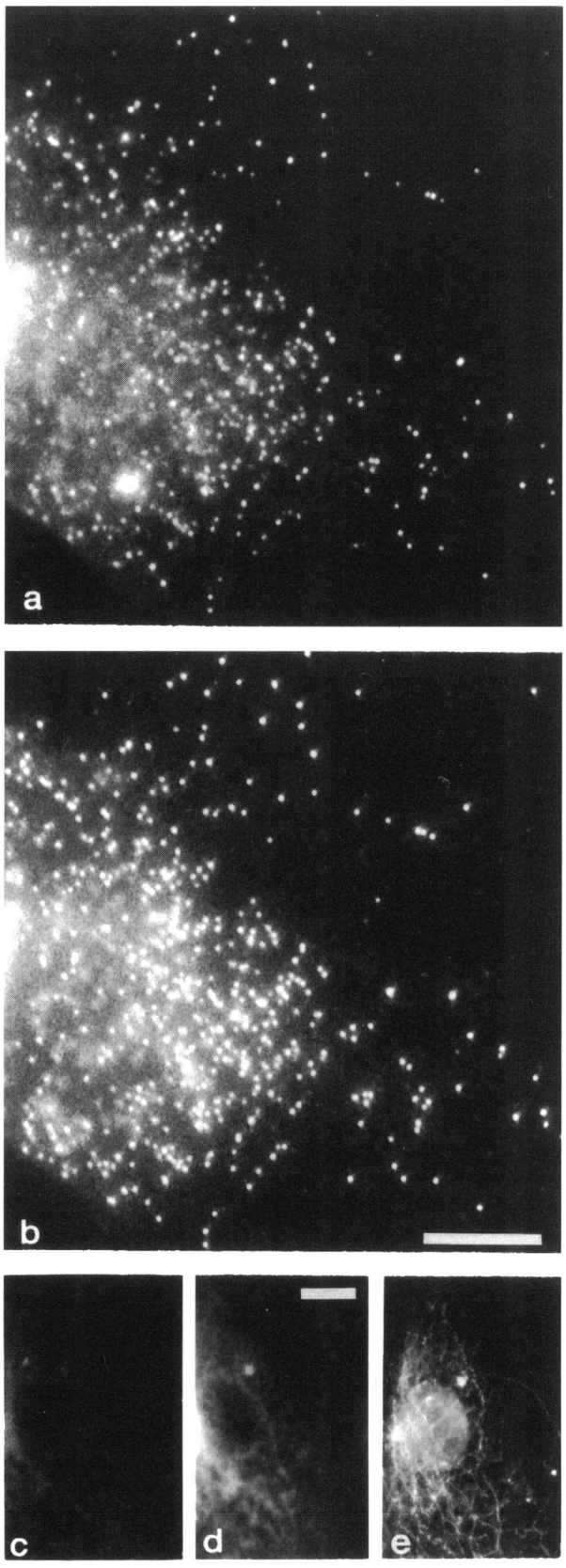

Figure 3. Peroxisomal import of IgG molecules bearing the peroxisomal targeting signal. Nonspecific rat IgG molecules, cross-linked with the synthetic peptide NH_3-CRYHLKPLQSKL-COOH were prepared as described in MATERIALS AND METHODS. After microinjection and incubation for 16 h at 37°C, cells were washed with PBS and permeabilized with digitonin (25 μg/ml) for 10 min. After the digitonin treatment the cells were fixed, permeabilized, and immunostained. This employed rabbit anti-catalase antibodies in the first step, and rhodamine-conjugated donkey anti-rat IgG and FITC-conjugated anti-rabbit IgG antibodies in the second step. The figure shows the intracellular localization of IgG-SKL molecules (a) and endogenous peroxisomes indicated by the presence of catalase (b). As a control to confirm that the IgG-SKL was within the peroxisomal matrix, omission of the Triton X-100 permeabilization step from the staining protocol resulted in the loss of the staining for IgG-SKL (c) and catalase (d), but not for β-tubulin (e). Bar, 5 μm.

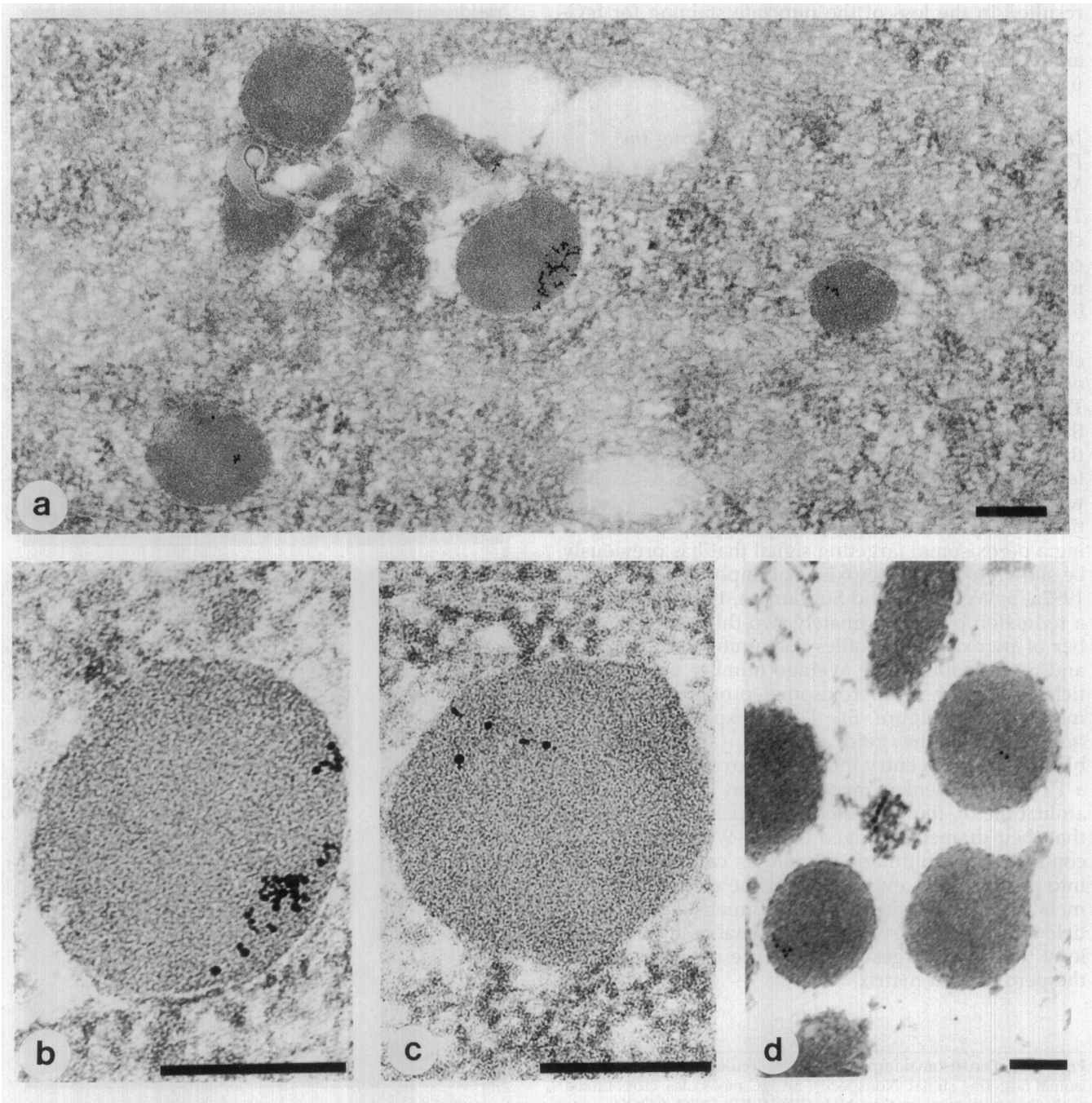

Figure 4. Peroxisomal import of gold particles bearing the SKL peroxisomal targeting signal. Colloidal gold particles (4–9 nm diameter), coated with bHSA-SKL were prepared as described in MATERIALS AND METHODS. After microinjection into the cytosol of Hs68 cells and incubation for 16 h at 37°C, cells were fixed and prepared for electron microscopy. Figure 4a shows the intracellular location of the microinjected gold particles. Microinjected gold/bHSA-SKL complexes were detected within electron-dense vesicles (b and c); these vesicles were confirmed to be peroxisomes by the presence of immunologically detectable catalase (d). Bar, 0.2 μm.

DISCUSSION

This study was undertaken to determine whether stably folded proteins were substrates for peroxisomal import. Our results support the conclusion that proteins in a stably folded conformation can be imported into the peroxisomal matrix via the PTS1-dependent pathway. These results extend recent observations that, in *S. cerevisiae*, multimeric protein complexes

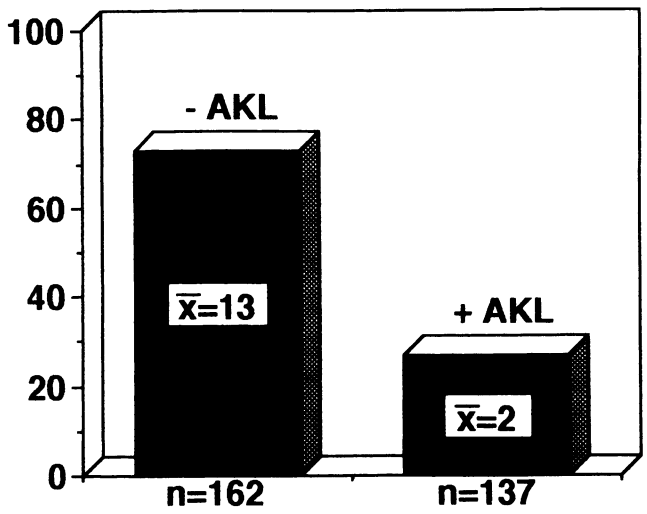

Figure 5. Effects of co-injection of a synthetic peptide bearing the Type I peroxisomal targeting signal (H_2N-CRYHLKPLQ AKL-COOH, 5 mg/ml) on the import of the gold/bHSA-SKL complex. Histogram depicts the fraction of peroxisomes observed bearing gold particles from experiments in the absence or presence of the co-injected peptide, "x" equals the average number of gold particles observed per peroxisomal profile, and "n" equals the number of peroxisomes observed for each condition.

could be imported into peroxisomes, even if one of the constituents of the complex lacked a peroxisomal targeting signal (Glover *et al.*, 1994; McNew and Goodman, 1994). These authors postulated that peroxisomal import occurred without requiring the unfolding of the complex to the point of dissociation. Our present results demonstrate that a protein-sized gold particle can gain access to the peroxisome by virtue of its association with a protein possessing a PTS, and that unfolding to an extended conformation is not required. The peroxisomal import of multimeric complexes and gold particles noncovalently associated with a peroxisomal protein (HSA-SKL) provides strong evidence that proteins lacking the PTS may be coimported into peroxisomes simply by their association with PTS-containing peroxisomal proteins. The import of such complexes had been proposed by Goodman's group several years ago, as judged by the stoichiometric association of the enzymes of methanol assimilation on the peroxisomal membranes under conditions that favored binding but not translocation (Bellion and Goodman, 1987).

Our findings do not rule out the existence of chaperones (Walton *et al.*, 1994) that facilitate import or assembly of peroxisomal proteins, either before or after translocation of proteins across the peroxisomal membrane. Indeed, the results of Frydman *et al.* (1992) on the folding of peroxisomal luciferase by the cytosolic chaperone TRiC, although not by the prokaryotic GroEL, may indicate an important biological function in vivo. As stably folded proteins can be imported into the matrix of the peroxisome, these proteins may make use of the cytosolic chaperones between the time of their synthesis and the time of their import. The demonstration of peroxisomal proteins in association with a cytosolic chaperone would confirm this functional interaction, and we are presently undertaking these experiments. However, if an unfolded conformation is not a requirement for peroxisomal import, what is the function of the cytosolic hsp70 molecules? As we have stated previously (Walton *et al.*, 1994), we believe that hsp70 molecules may retard the folding of the PTS until it can interact with the appropriate receptor molecule, and thereby facilitate import kinetically.

Do these present results indicate that all of the constituents of the peroxisomal matrix form in the cytosol before import? One line of experimental results indicate that this is not true for all matrix proteins. Investigators have demonstrated that catalase can form enzymatically active tetrameric complexes in the cytosol of fibroblasts from patients with Zellweger syndrome (Wanders *et al.*, 1984). Catalase appears to redistribute to peroxisomes after fusion of complementary cell lines (Brul *et al.*, 1988), and this import can be inhibited by aminotriazole, which appears to prevent unfolding of the protein complex (Middelkoop *et al.*, 1991). Thus, it appears that the 240-kDa catalase tetramer is not a substrate for peroxisomal import. Whether this is due to the size of the tetramer, or due to the stabilization of a conformation that does not display a PTS cannot be resolved at this time.

The translocation of folded proteins and protein complexes into the peroxisome is quite unlike the process of import across the membranes of the mitochondrion or chloroplast. It is possible that organelles that possess ionic or chemical gradients across their membranes require a more restrictive form of protein translocation. The ability of peroxisomes to import proteins without requiring unfolding is reminiscent of the nuclear pore. Pore-like structures have never been described in morphological studies of the peroxisomal membrane. However, the mammalian peroxisomal membrane has been shown to possess pores by electrophysiological (Labarca *et al.*, 1986; Lemmens *et al.*, 1989), and solute permeability measurements (Nicolay *et al.*, 1987; Van Veldhoven *et al.*, 1987). The estimated diameter of these peroxisomal pores from the electrophysiological data is between 1.5–3 nm. Although this is less than the 4-nm diameter of HSA or the 9-nm diameter of the gold particles, the nuclear pore can open to accommodate larger proteins if

they possess the correct targeting signal. Whether the peroxisomal pores previously described have a function in the import of matrix proteins remains to be examined.

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