Translocation of Acyl-CoA Oxidase into Peroxisomes Requires ATP Hydrolysis but Not a Membrane Potential

Tsuneo Imanaka, Gillian M. Small, and Paul B. Lazarow

The Rockefeller University, New York 10021

Abstract. An efficient system for the import of newly synthesized proteins into highly purified rat liver peroxisomes was reconstituted in vitro. ^{35}S-Labeled acyl-CoA oxidase (AOx) was incorporated into peroxisomes in a proteinase K–resistant fashion. This import was specific (did not occur with mitochondria) and was dependent on temperature, time, and peroxisome concentration. Under optimal conditions ~30% of [^{35}S]AOx became proteinase resistant. The import of AOx into peroxisomes could be dissociated into two steps: (*a*) binding occurred at 0°C in the absence of ATP; (*b*) translocation occurred only at 26°C and required the hydrolysis of ATP. GTP would not substitute for ATP and translocation was not inhibited by carbonylcyanide-*m*-chlorophenylhydrazone, valinomycin, or other ionophores.

The biogenesis of peroxisomes has unique features that distinguish it from the assembly of other organelles (21). In rat liver, for example, the peroxisomal matrix proteins, the core protein urate oxidase, and a major 22-kD integral membrane protein are all synthesized on free polyribosomes. Newly synthesized polypeptides are initially located in the cell cytosol and subsequently appear in peroxisomes, as shown by in vivo pulse-chase experiments. These findings imply the posttranslational import of newly synthesized polypeptides by preexisting peroxisomes. In this respect, peroxisome assembly resembles that of mitochondria and chloroplasts, although peroxisomes have only one membrane. In contrast to most other organelle proteins, peroxisomal proteins are generally synthesized at their final sizes and are not processed proteolytically upon import. Moreover, topogenic information is present in the carboxy-terminal region of the one peroxisomal protein so far studied (38).

Practically no information is yet available on the details of the import mechanism, including the energy requirements (21). Bellion and Goodman have recently reported (2) that carbonylcyanide-*m*-chlorophenylhydrazone (CCCP)[1] prevents the import of alcohol oxidase into *Candida boidinii* peroxisomes. This effect is puzzling in view of the fact that peroxisomal membranes contain pores that allow the passage of molecules as large as 800 D (40). A requirement for ATP has emerged recently as a common factor in posttranslational protein translocation into mitochondria, chloroplasts, and endoplasmic reticulum (see Discussion). In view of the differences in peroxisome biogenesis noted above, it is unpredictable whether or not peroxisomes might also require ATP for protein import.

An efficient in vitro reconstitution of the import of polypeptides into peroxisomes would permit investigation of the molecular mechanism. Such experiments face the obstacles that peroxisomes are fragile and represent only 2.5% of the normal rat liver cell protein. A limited in vitro import of acyl-CoA oxidase (AOx) and catalase into rat liver peroxisomes was accomplished (11), but the efficiency was too low to serve as a routine assay.

Here we report major improvements in the import assay. mRNA was isolated from livers of rats treated with clofibrate, a hypolipidemic drug that strikingly induces peroxisome proliferation (17, 32), peroxisomal enzymes (20, 22), and mRNAs that encode peroxisomal enzymes (13, 24, 31). In particular, clofibrate induces 15-fold the mRNA encoding AOx, the first enzyme in the peroxisomal fatty acid β-oxidation system (24). Stable rat liver peroxisomes were purified in Nycodenz gradients. With the inclusion of $MgCl_2$ and KCl in the medium, efficient import of AOx was obtained (~30%). This improved system permitted us to study the energy requirements for AOx import: ATP hydrolysis was necessary but, in contrast to the results of Bellion and Goodman (2), CCCP and other ionophores had no effect.

Portions of this work have appeared in abstract form (1986. *Eur. J. Cell Biol.* 41[Suppl. 14]: 20; 1986. *J. Cell Biol.* 103[5, pt. 2]:524*a*. [Abstr.]).

1. *Abbreviations used in this paper*: AMP-PNP, adenosine-5′-[β,γ-imido]-triphosphate; AOx, acyl-CoA oxidase; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; HD, bifunctional hydratase–dehydrogenase; SEH, 0.25 M sucrose, 0.1% (vol/vol) ethanol, 5 mM Hepes/KOH, pH 7.4; SVEH, 0.25 M sucrose, 1 mM EDTA, 0.1% (vol/vol) ethanol, 5 mM Hepes/KOH, pH 7.4.

Materials and Methods

Purification and Concentration of Peroxisomes or Mitochondria

Peroxisomes were purified by sequential differential and isopycnic centrifugation from three female F-344 rats (150–200 g) according to Leighton et

© The Rockefeller University Press, 0021-9525/87/12/2915/8 $2.00

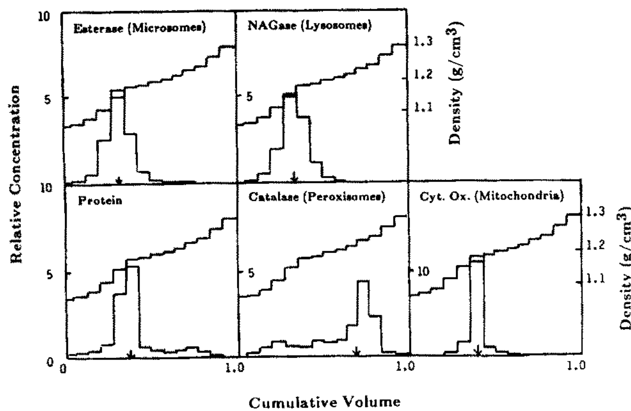

Figure 1. Isolation of peroxisomes in Nycodenz. A light mitochondrial (lambda) fraction from normal rat liver (7.5 ml, ~30 mg protein/ml) was fractionated by equilibrium density centrifugation. The normalized marker enzyme distributions are plotted as described (23); the recoveries varied between 70 and 113%. *NAGase*, *N*-acetyl-β-D-glucosaminidase; *Cyt. Ox.*, cytochrome *c* oxidase. The arrows indicate the medians of the distributions.

al. (23) except that (*a*) the rats were not pretreated with Triton WR-1339; (*b*) the homogenization medium was 0.25 M sucrose, 1 mM EDTA, 0.1% (vol/vol) ethanol, pH 7.4 (SVE); (*c*) the density gradient (1.15-1.25 g/ml) was formed with Nycodenz (Nyegaard Co., Oslo, Norway); and (*d*) the gradient rested on a 6 ml cushion of Nycodenz (1.3 g/ml). All Nycodenz solutions contained SVE and 5 mM Hepes/KOH, pH 7.4 (SVEH). As shown in Fig. 1, the peroxisomes (marker enzyme, catalase) were well separated from mitochondria, microsomes, and lysosomes. As viewed by electron microscopy, they were nearly homogeneous (Fig. 2 *a*). The purity of the peroxisome fraction was calculated from the relative specific activities of the marker enzymes to be ~95% (Table I).

To remove Nycodenz and concentrate the peroxisomes, gradient fractions were pooled, slowly diluted by the dropwise addition of 5 vol of ice-cold SVEH, and centrifuged for 20 min at 12,000 rpm (17,000 g; SS-34 rotor; Sorvall Instruments Div., Du Pont de Nemours & Co., Inc., Newtown, CT). The pellet was resuspended in 0.25 M sucrose, 0.1% (vol/vol) ethanol, 5 mM Hepes/KOH, pH 7.4 (SEH) to a final protein concentration of ~10 mg/ml using a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) with a type B (tight) pestle. Approximately 70% of catalase and protein were recovered in the concentrated organelles (Table II). Morphologically (Fig. 2 *b*), the peroxisomes appear to contain less matrix material than when first isolated, but their membranes are apparently intact. The peroxisomes could be rediluted and reconcentrated without appreciable further loss of catalase (not shown).

Mitochondria from the Nycodenz gradient (80-85% pure) were similarly diluted and concentrated except that centrifugation was at 6,300 rpm (4,500 *g*) for 12 min. The mitochondria contained 2% peroxisomal contamination. A cytosol fraction was prepared by centrifugation of a rat liver postnuclear supernatant at 40,000 rpm for 1 h in a 50Ti rotor (150,000 *g*; Beckman Instruments, Inc., Palo Alto, CA).

Isolation and Translation of RNA

Total RNA was isolated by guanidinium thiocyanate/guanidinium chloride extraction (6) from the livers of rats that were given clofibrate (5 g/kg chow) for 2 wk. Total RNA extracted with phenol/chloroform (36) from a post-mitochondrial fraction of liver from a clofibrate-treated rat was used in some experiments. RNA (final concentration 200-600 μg/ml) was translated with [^{35}S]methionine (1,000 Ci/mmol) at 29°C for 90 min in a nuclease-treated reticulocyte lysate protein-synthesizing system (27).

Posttranslational Import Assay

After in vitro translation, the translation mixture was centrifuged in a 50Ti rotor (Beckman Instruments, Inc.) at 40,000 rpm (150,000 *g*) for 1 h to remove polyribosomes. 2 M sucrose was added to the supernatant to a final concentration of 0.25 M. In a typical import experiment 25 μl of the adjusted translation mixture was mixed with 25 μl of purified, concentrated peroxisomes (200-240 μg protein, except as noted) and 50 μl of SEH. In later experiments (Figs. 7-10) 3 mM $MgCl_2$ and 50 mM KCl (final concentrations) were also added. After incubation at 26°C for 30 min, the import mixture was diluted 10-fold with ice-cold SVEH containing 1 mM methionine. Nonimported translation products were digested with 4 μg of proteinase K at 0°C for 15 min; the reaction was terminated with 10 μl of protease inhibitor mixture (10 mM phenylmethylsulfonyl fluoride, 12.5 mM *N*-tosyl-L-lysyl-chloromethylketone, 7 mU/ml of aprotinin [trypsin inhibitor units], 4 mM leupeptin, 4 mM chymostatin, 4 mM antipain, and 4 mM pepstatin). A duplicate import assay received protease inhibitors only. The samples were then centrifuged for 10 min in a microcentrifuge (16,000 *g*; No. 5414; Eppendorf) to reisolate the peroxisomes, which were solubilized with SVE containing 1% (wt/vol) sodium deoxycholate, 1% (wt/vol) Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride. 25% of the pellets and 2.5% of some supernatants were subjected to SDS-PAGE (12) and fluorography on preflashed film (19); the exposure time of the supernatants was 10

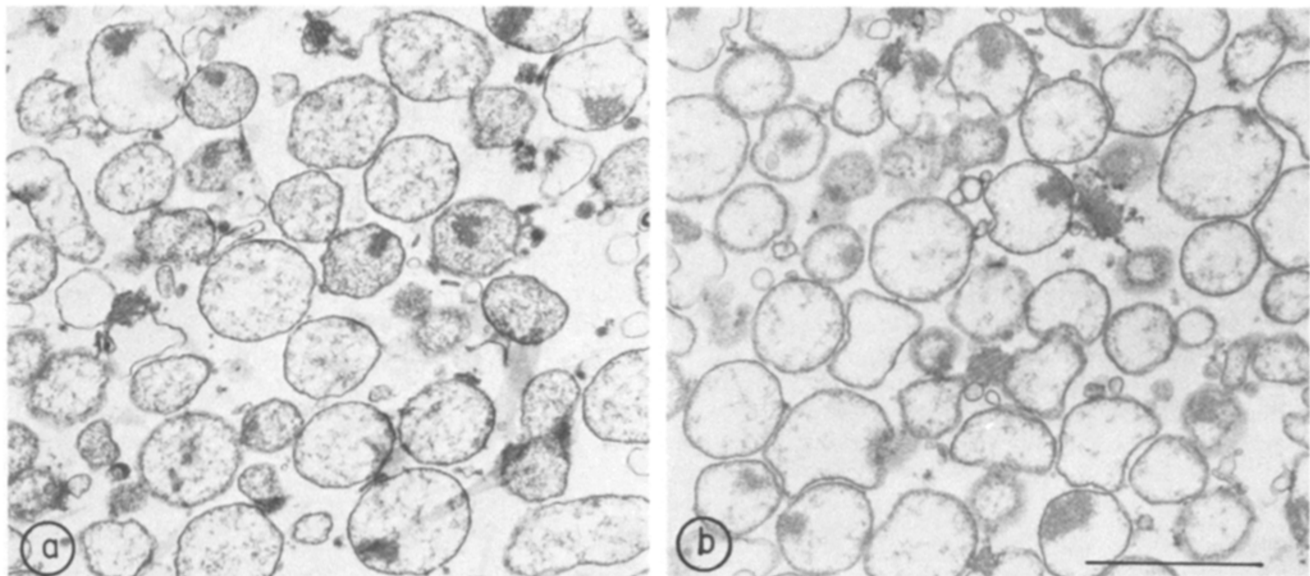

Figure 2. Electron micrograph of Nycodenz-purified peroxisomes from normal rat liver. (*a*) Peroxisomes from the Nycodenz gradient. (*b*) Concentrated peroxisomes after dilution and centrifugation. Bar, 1 μm.

Table I. Isolation of Peroxisomes by Nycodenz Gradient Centrifugation

Constituent	Marker for	Homogenate activity	Light mitochondrial fraction	Peak peroxisomal fraction from isopycnic gradient	
		U/g liver	*% of homogenate*	*relative specific activity*	*% purity (% contamination)**
Protein		243	4.68		
Catalase	Peroxisomes	61.2	35.9	39.4	∼95
Cytochrome oxidase	Mitochondria	9.76	18.0	0.051	(1.02)
Esterase	Endoplasmic reticulum	190	2.80	0.163	(3.26)
NAGase	Lysosomes	812	32.6	0.377	(0.74)

* Calculated (see reference 12) from the relative specific activities, assuming that mitochondria and endoplasmic reticulum each represent 20% of liver protein and lysosomes represent 2.0% of liver protein (23).

Table II. Removal of Nycodenz and Concentration of Peroxisomes

	Catalase	Protein	Specific activity
	U	*mg*	*U/mg*
Peroxisomes in Nycodenz	76.3	10.2	7.51
Supernatant	11.4	2.58	4.38
Concentrated peroxisomes	57.2	6.94	8.25
Recovery	89.9%	93.3%	

The peroxisome fractions from the Nycodenz gradient were pooled and diluted fivefold with SVEH. The peroxisomes were pelleted by centrifugation and resuspended in SEH at ∼10 mg/ml.

times longer than that of the pellets. To quantitate import, 10–50% of the adjusted translation products were mixed with peroxisomes (200–240 μg as above) and 25% of the mixture was analyzed by SDS-PAGE and fluorography, as for the peroxisome pellets. Radioactivity was quantitated by scanning densitometry with a densitometer (model 1650; Bio-Rad Laboratories, Richmond, CA); generally more than one exposure of each fluorogram was scanned and it was verified that the exposures used were in the range that gave a linear response.

Other Methods

Protein was determined with the protein assay kit (Bio-Rad Laboratories) with bovine-γ-globulin as standard (3). *N*-acetyl-β-D-glucosaminidase was assayed according to Findlay et al. (9). Other marker enzymes and refractive indices were measured as before (1). ATP was assayed with a bioluminescence CLS kit (Boehringer Mannheim Diagnostics, Inc., Houston, TX) after deproteinization with trichloroacetic acid.

Samples for electron microscopy were fixed with 2% glutaraldehyde (final concentration) in 0.1 M cacodylate buffer (pH 7.4) overnight and pelleted by centrifugation at 45,000 rpm for 30 min in a 50Ti rotor (Beckman Instruments, Inc.). The pellets were postfixed with 1% OsO_4 and processed for electron microscopy as described previously (1).

Results

Peroxisome Stability

Peroxisome stability is critically important to an assay of posttranslational protein import. Stability was measured as the latency of catalase (the percentage of the total catalase activity that is not assayable when the peroxisomal membrane is intact, and which appears when the membrane is disrupted by detergents). In the concentrated, purified peroxisomes, the latency of catalase was ∼90% and it did not decrease during incubation at 26°C for 90 min or at 0°C for 3 h (Table III).

The peroxisomes could be incubated with as much as 1/4 their weight of proteinase K for 20 min at 0°C without loss of latency (Table IV). Routinely, peroxisomes were treated at 0°C with 5% of their weight of proteinase K after the import incubation. This sufficed to digest most nonimported [^{35}S]translation products (Fig. 3, lane *3*) without affecting the SDS-PAGE pattern of peroxisome content proteins.

Table III. Maintenance of Catalase Latency during Incubation

Incubation		Catalase activity		
Temperature	Time	Total (+ Triton X-100)	Free (− Triton X-100)	Catalase latency*
°C	*min*	*U/ml*	*U/ml*	*%*
26	0	28.4	2.24	92
	30	33.4	2.60	92
	60	39.1	3.45	91
	90	27.1	2.46	91
0	90	33.7	2.57	92
	180	26.7	2.81	89

Concentrated peroxisomes (2 mg/ml) were incubated in SEH. At the times indicated, a sample was removed and diluted 200-fold with SEH. Catalase activity was measured at 0°C for 5 min in the presence of 0.25 M sucrose.
* Catalase latency is (total activity − free activity)/total activity × 100%.

Table IV. Stability of Peroxisomes to Treatment with Proteinase K

Proteinase K	Catalase activity Total (+ Triton X-100)	Free (− Triton X-100)	Catalase latency
μg	*U/ml*	*U/ml*	*%*
0	26.7	2.81	89
8	31.4	3.66	88
20	30.6	3.20	90
40	25.9	3.09	88

Concentrated peroxisomes (160 μg) in 40 μl of SEH were incubated with proteinase K at 0°C for 20 min. After incubation, the peroxisomes were diluted 100-fold with SEH containing 0.2 mM phenylmethylsulfonyl fluoride and catalase activity was measured (at 0°C for 5 min). 4 μg of proteinase K (in a total volume of 1 ml) was used in subsequent routine experiments.

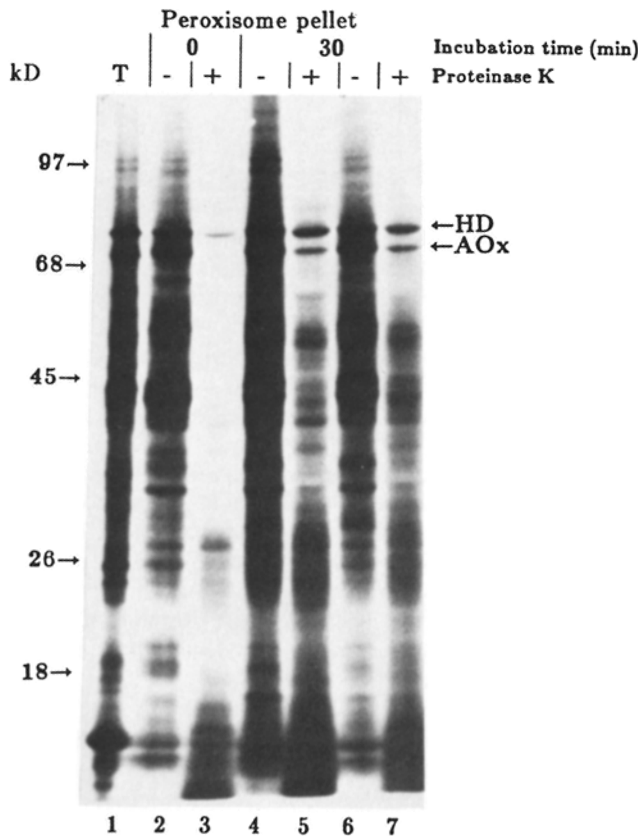

Figure 3. Posttranslational import assay. A postribosomal supernatant of the cell-free translation mixture (25 μl) was mixed at 0°C with rat liver peroxisomes (200 μg) in a volume of 100 μl of SEH as described in Materials and Methods. Immediately, or after incubation at 26°C for 30 min, the import mixture was diluted and duplicate samples were (+) or were not (−) treated with proteinase K at 0°C for 15 min. The peroxisomes were pelleted by centrifugation and analyzed by SDS-PAGE and fluorography. Arrows at the left side indicate the molecular mass standards. *HD*, peroxisomal bifunctional hydratase-dehydrogenase; *AOx*, peroxisomal acyl-CoA oxidase; *T*, 17% of the total translation products used for import. In lanes *6* and *7*, rat liver cytosol (high speed supernatant, 970 μg of protein) was added to the import assay.

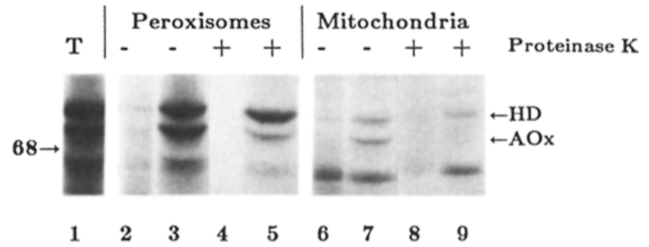

Figure 4. Immunoprecipitation of acyl-CoA oxidase; organelle specificity. The import assay was carried out with equal amounts of peroxisomal or mitochondrial protein. AOx was immunoprecipitated from one-fourth of each organelle pellet with a polyspecific goat antiserum (lanes *3*, *5*, *7*, and *9*) or preimmune serum (lanes *2*, *4*, *6*, and *8*). Lane *1*, immunoprecipitation of total translation products (2 μl). This polyspecific antiserum recognizes HD as well as AOx, and to a lesser extent, other peroxisomal proteins (30). The lack of digestion of HD by proteinase K in lanes *5* and *9* does not imply import because HD is intrinsically resistant to proteinase K (see text). Because these immunoprecipitations used a goat serum, we omitted protein A (11) and included 50 μg of peroxisomal protein as carrier with the mitochondria and total translocation products.

Posttranslational Import of Proteins into Peroxisomes

Many ^{35}S-labeled polypeptides sedimented with peroxisomes after a cell-free translation mixture was incubated with the peroxisomes at 26°C for 30 min (Fig. 3, lane *4*). A portion of these are resistant to proteinase K (Fig. 3, lane *5*), indicating that they have been imported into the peroxisomes. In the nonincubated control binding to the peroxisomes was much less, and protease resistance was almost nil (Fig. 3, lanes *2* and *3*). The addition of a rat liver high speed supernatant (cytosolic) fraction to the import assay mixture had little effect on import (Fig. 3, lane *7*), but did reduce binding of some nonimported proteins (lane *6*).

Among the proteinase K-resistant translation products, the prominent doublet of 72 and 78 kD corresponds (30) to two peroxisomal proteins involved in fatty acid oxidation: AOx and the bifunctional hydratase–dehydrogenase (HD), respectively. The identification was confirmed by immunoprecipitation (Fig. 4, lane *1*). These proteins are prominent among the total translation products due to the fact that the rats had been treated with the peroxisome-proliferating agent, clofibrate (30).

The protease resistance of most of the translation products in Fig. 3, lane *5* was abolished by detergents (not shown here, see below, Figs. 7 and 9). HD was an exception in that proteolysis of detergent-solubilized HD and of nonimported HD (see below, Fig. 6, *Supernatant*) was variable and often incomplete, consistent with previous results (11). Therefore the present experiments do not distinguish between binding and import of HD.

We focused our subsequent experiments on AOx because: (*a*) it is a major translation product; (*b*) it is easily digested by proteinase K; (*c*) it is readily distinguished in the SDS gel; and (*d*) it may be quantitated by densitometric scanning of autoradiograms without immunoprecipitation in routine experiments.

Characterization of AOx Import

To determine the organelle specificity of AOx import, con-

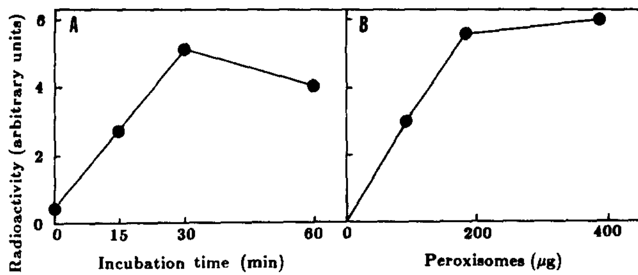

Figure 5. Time dependence and effect of peroxisome concentration on the import of AOx. Peroxisomal pellets were analyzed by SDS-PAGE and fluorography. Proteinase K-resistant [^{35}S]AOx was quantitated by scanning densitometry. Radioactivity is expressed as the area of each peak in arbitrary units. (*A*) 186 μg of peroxisomal protein was used. (*B*) 30-min incubation.

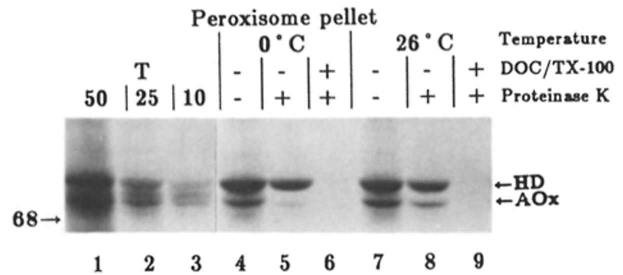

Figure 7. Temperature dependence of import. The import assay was carried out with KCl and $MgCl_2$ at 26° or 0°C for 30 min. In lanes *6* and *9*, the import mixture was treated with 1% sodium deoxycholate and 1% Triton X-100 prior to proteinase K digestion. *T*, total translation products corresponding to 10, 25, and 50% of the input radioactivity, subjected to coelectrophoresis with peroxisomal proteins.

trol experiments were carried out with mitochondria. Very little of the [^{35}S]AOx translation product appeared in the organelle pellet, and none was protease resistant (Fig. 4). Likewise, mitochondria do not appreciably bind HD (Fig. 4, lane *7* vs. lane *3*). In a mock import experiment without organelles, no AOx appeared in the pellet (not shown).

The import of [^{35}S]AOx into peroxisomes increased linearly with time up to 30 min (Fig. 5 *A*) and was maximal with 200 μg of peroxisomal protein (Fig. 5 *B*).

$MgCl_2$ (3 mM) and KCl (50 mM) markedly stimulated the import of the AOx translation product into peroxisomes (Fig. 6 *A*). Protease-resistant [^{35}S]AOx increased from ~6% in the control (Fig. 6 *A*, lane *5*) to 30% of the added [^{35}S]AOx (lanes *7*, *9*, and *11*). Total [^{35}S]AOx associated with the organelles was not affected and represented ~60% of the added protein (Fig. 6 *A*, lanes *4*, *6*, *8*, and *10*). The ions did not affect the digestibility of AOx by proteinase K, as seen in the supernatants (Fig. 6 *B*) and in the nonincubated peroxisome pellet (Fig. 6 *A*, lane *3*). The effects of KCl and $MgCl_2$ were not additive. NaCl was almost as effective as KCl (not shown). In subsequent import experiments, 3 mM $MgCl_2$ and 50 mM KCl were added routinely.

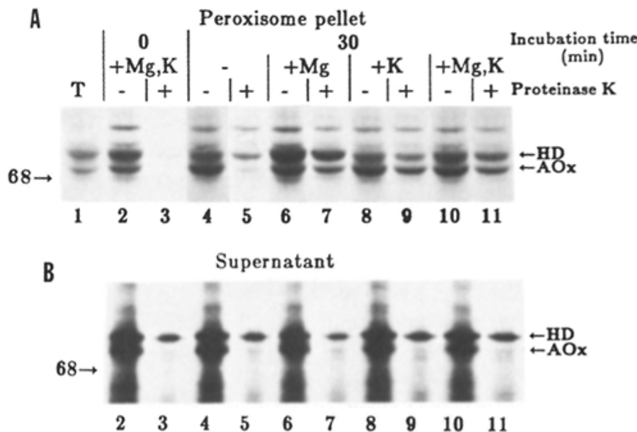

Figure 6. Effect of $MgCl_2$ and/or KCl on the import of AOx. $MgCl_2$ (3 mM) and/or 50 mM KCl were added to the import assay where indicated (lanes *4–11*). In lanes *2* and *3*, the import mixtures were analyzed immediately after mixing translation products and peroxisomes. *T*, 25% of the translation mixture used in the import assay, subjected to coelectrophoresis with peroxisomal protein. Binding (*even* lanes) was quantitated on a shorter fluorographic exposure than shown here.

When the import incubation was carried out at 0°C, binding of [^{35}S]AOx to peroxisomes was similar to that at 26°C, but proteinase K-resistant [^{35}S]AOx was negligible (Fig. 7). This demonstrates that binding of AOx to peroxisomes may be dissociated from translocation of AOx into the organelle; only the latter step is temperature dependent. The experiment also verifies that AOx bound to the outside of peroxisomes is digested by proteinase K.

AOx Import Requires ATP Hydrolysis but Not a Membrane Potential

ATP derived from the mRNA translation mixture is present in the routine import assay. The ATP-regenerating system added to the reticulocyte lysate maintains the ATP concentration during translation: we measured 1.1–1.2 mM ATP (n = 7) at the end of 90 min of translation. Diluted four times in the peroxisome import assay, this gave a concentration of ~0.3 mM ATP during import. The addition of a further 1 mM ATP did not improve the efficiency of import of AOx (not shown). Depletion of ATP with apyrase or hexokinase plus glucose (Fig. 8 *A*) markedly reduced the import of AOx (Fig. 8 *B*). Treatment of the translation mixture with heat-inactivated enzymes had no effect.

The protonophores, CCCP and 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF6847), had no effect on the import of AOx into peroxisomes (Fig. 9). Likewise, the ionophores nigericin, valinomycin, and monensin had no effect. Protease-protected [^{35}S]AOx in the presence of the ionophores varied between 19 to 27% of added [^{35}S]AOx in comparison with 18 and 22% protection in two controls.

The translation mixture was also depleted of ATP by centrifugation through Sephadex G-25 (Table V). Import of [^{35}S]AOx into peroxisomes was thereby reduced from 30 to 3.5% (Fig. 10). The addition of 1.0–1.3 mM ATP restored AOx import to 12–16% (Table V). GTP (1 mM) or 1 mM of the nonhydrolyzable analogue of ATP (adenosine-5′-[β,γ-imido]triphosphate, [AMP-PNP]) were not able to restore import (Fig. 10). Moreover, in a similar experiment, in which 0.3 mM ATP was added after Sephadex G-25 chromatography, the import was inhibited by the concomitant addition of 1 or 3 mM AMP-PNP (data not shown). This

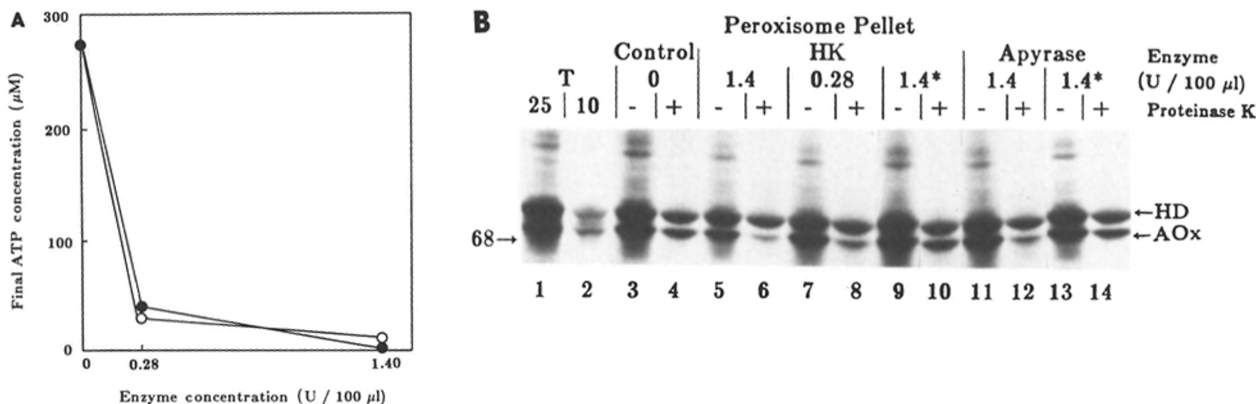

Figure 8. Depletion of ATP from the import assay. (*A*) ATP. The cell-free translation products (25 μl) were diluted fourfold in SEH, 50 mM KCl, 3 mM $MgCl_2$ as for an import assay and incubated with apyrase (grade VI; Sigma Chemical Co., St. Louis, MO) (*open circles*) or hexokinase (type VI; Sigma Chemical Co.) plus 10 mM glucose (*solid circles*) at 26°C for 10 min. (*B*) Import. Peroxisomes were added to cell-free translation products from which ATP had been depleted as in *A* (lanes *5–8*, *11*, and *12*). As controls, peroxisomes were similarly added to import mixtures preincubated without ATP-depleting enzymes (lanes *3* and *4*) or to mixtures preincubated with heat-inactivated enzymes (100°C, 5 min) (*asterisks*, lanes *9*, *10*, *13*, and *14*). *T*, total translation products as in Fig. 7.

competition of the nonhydrolyzable analogue with ATP demonstrates that AMP-PNP binds to the ATP-utilizing enzyme. It thus serves as a positive control for the inability of AMP-PNP to support import.

The total amount of [^{35}S]AOx associated with peroxisomes in the experiment of Fig. 10 was 20–28% of that added, regardless of the presence or absence of ATP (Table V). Therefore the precise location of some 9–13% of the added [^{35}S]AOx (Table V, difference between 3–3.5 and 12–16%) depends on the ATP concentration: in the absence of ATP it is bound to the surface of the peroxisome and is digestible by proteinase K; in the presence of ATP it is inside the organelle and protected from proteinase K.

Discussion

We have markedly improved the conditions for measuring the in vitro import of peroxisomal proteins into rat liver peroxisomes. This has permitted us to investigate two steps in the process (binding and translocation) and to determine the energy requirements. Chief among the improvements were (*a*) the use of Nycodenz as the density gradient material for peroxisome isolation. Nycodenz has 20% of the osmolality that sucrose has for the same density, lessening the likelihood of rupturing the peroxisomes when they are diluted and concentrated. Furthermore, lysosomes (a major contaminant of peroxisomes in sucrose gradients unless rats are pretreated with Triton WR-1339) (23) are well separated from peroxisomes in Nycodenz gradients (see Materials and Methods). (*b*) Rats used for isolation of liver mRNA were pretreated with clofibrate, thus inducing mRNAs encoding peroxisomal enzymes (13, 24, 31). This permits the cell-free translation product corresponding to the peroxisomal β-oxidation enzyme AOx to be easily recognized and quantitated without the need for routine immunoprecipitation (30). (*c*) 50 mM KCl and 3 mM $MgCl_2$ were included in the import buffer.

Two experimental approaches allowed us to distinguish binding from translocation. When the import assay was carried out at 0° instead of 26°C, just as much [^{35}S]AOx became associated with the peroxisomes, but it all remained on the surface, accessible to digestion by proteinase K. This binding to the peroxisomal membrane was specific in that [^{35}S]AOx did not appreciably bind to mitochondria, even at 26°C (the limited amount of [^{35}S]AOx seen in the pellet in Fig. 4, lane *7* may even reflect the measured 2% contamination of the mitochondrial fraction by peroxisomes). The fact that one-third to one-half of the [^{35}S]AOx present on the peroxisome membrane at 0°C would have been internalized

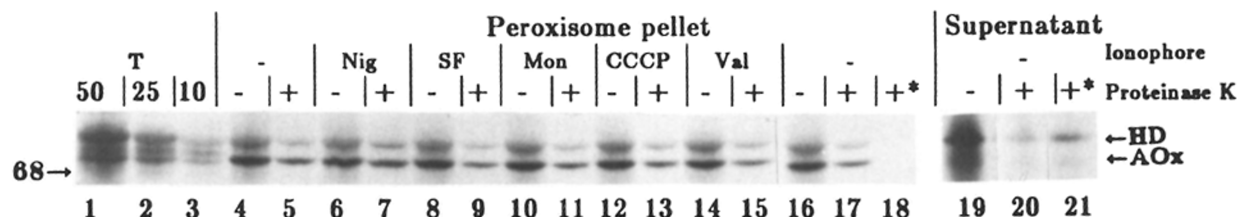

Figure 9. Addition of ionophores to the import assay. Import was carried out in the presence of the following ionopnores (10 μM each): none (lanes *4*, *5*, and *16–18*), nigericin (lanes *6* and *7*), SF6847 (lanes *8* and *9*), monensin (lanes *10* and *11*), CCCP (lanes *12* and *13*), and valinomycin (lanes *14* and *15*). The ionophores were added to each 100 μl import assay in 1 μl of ethanol. As a control, the assay was carried out with 1 μl of ethanol (lanes *4* and *5*) and without ethanol (lanes *16–18*). 1% deoxycholate and 1% Triton X-100 were added to the experiment of lane *18* just before proteinase K digestion (*). Each peroxisomal pellet (lanes *4–18*) and the supernatants from one control (lanes *19–21*) were analyzed by SDS-PAGE and fluorography.

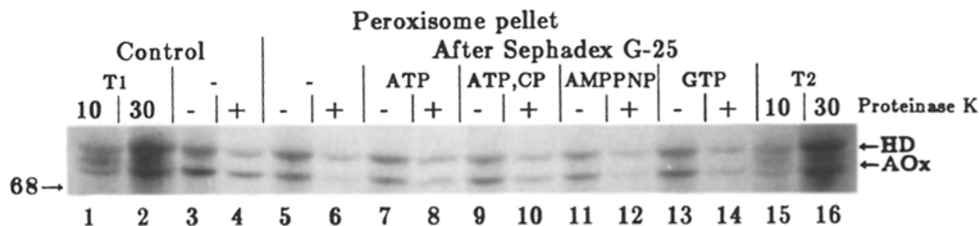

Figure 10. Addition of triphosphates to an import assay depleted of ATP by gel filtration. Translation products (350 μl) were centrifuged (1,600 *g*, 4 min, 4°C) through a 3.5-ml column of Sephadex G-25 (fine) equilibrated with 25 mM Hepes/KOH (pH 7.4), 1.5 mM $Mg(Ac)_2$, 65 mM KCl, and 2 mM dithiothreitol. (Just before use, the column was washed twice with 1.5 ml of 0.5% BSA in the above buffer.) Import was then carried out as usual. Additions were: none (lanes *5* and *6*), 1 mM ATP (lanes *7* and *8*), 1 mM ATP, 6 mM creatine phosphate, 5 μg of creatine phosphokinase (lanes *9* and *10*), 1 mM adenosine-5′-[β,γ-imido]triphosphate (AMP-PNP) (lanes *11* and *12*), or 1 mM GTP (lanes *13* and *14*). Nongel-filtered translation products were used for the import assay of lanes *3* and *4* without further addition of triphosphates. *T1*, nongel-filtered translation products; *T2*, gel filtered translation products; in both cases 10 and 30% of the input radioactivity was subjected to coelectrophoresis with peroxisomal proteins.

at 26°C strongly suggests that this is a functional intermediate in the import process. This interpretation is supported by the fact that ATP depletion produced exactly the same result. As shown by the quantitation in Table V, in the absence of ATP, membrane-bound [^{35}S]AOx/internalized [^{35}S]AOx was 17–24: 3–4%. In the presence of ATP the values were 10–11: 12–16%. The total amount of [^{35}S]AOx associated with peroxisomes was the same in both cases (20–28 or 23–26%); only the location in the organelle was different.

We do not know to what structure(s) on the peroxisomal membrane the [^{35}S]AOx bound, nor do we know that all of the binding is functional (will lead to import). In the case of mitochondrial protein import, functional specific binding to a receptor and unspecific binding to the membrane have been suggested (29) as has binding to the lipid bilayer followed by migration to a receptor (5, 41). Recent experiments indicate that mild proteolytic pretreatment of the peroxisomes reduces both binding and translocation (Imanaka, T., C. S. Redwood, H. Shio, G. M. Small, and P. B. Lazarow, manuscript in preparation), suggesting that at least some of the [^{35}S]AOx may be bound to a receptor. Future experiments will address in detail the nature and site of this binding.

The peroxisomal membrane is known to be very permeable, rather like the mitochondrial outer membrane, at least in rat liver (7). Sucrose and substrates such as glycolate, urate, and amino acids (7), as well as NAD^+, ATP, and coenzyme A permeate the membrane through a proteinaceous pore (18, 39, 40). These facts make it unlikely that this membrane could support a membrane potential or ion gradient. Therefore, the energy to drive the import process would be expected to come from another source. Indeed, we found that a variety of ionophores were without effect on import. ATP hydrolysis, on the other hand, was essential, and a concentration of 300 μM ATP gave maximal import.

These results differ from those of Bellion and Goodman (2), who reported that CCCP prevented the import of alcohol oxidase into peroxisomes of *Candida boidinii*. Although a species difference can not be excluded, this conflict is likely to reflect the difference in methods used. In the work of Bellion and Goodman, import was investigated in spheroplasts, and the CCCP was applied to the living cells. As noted by those authors, CCCP apparently caused a drastic drop in the intracellular ATP concentration. We suggest that it was this drop in ATP that prevented the import of alcohol oxidase rather than a direct effect of the CCCP on the peroxisomal membrane.

These results demonstrate that protein translocation into peroxisomes resembles, in one key respect (the ATP requirement), the posttranslational import of proteins into other organelles. In that ATP is needed but a membrane potential is not, peroxisomal import most closely resembles the import of proteins into chloroplasts (10, 15, 26, 35) and yeast microsomes (16, 33, 42), or the insertion into dog pancreas microsomes (25). It differs from translocation into mitochondria

Table V. Readdition of Triphosphates to ATP-depleted Import Assay

Translation mixture used for import	Additions	[^{35}S]AOx associated with peroxisomes*	[^{35}S]AOx imported into peroxisomes‡	[^{35}S]AOx bound to surface of peroxisomes§	ATP‖
			% of added [^{35}S]AOX		*μM*
Before Sephadex	None	39	30	9	275
After Sephadex¶	None	28	3.5	24	0
	ATP, 1 mM	23	12	11	1,000
	ATP, CP, CPK**	26	16	10	1,300
	AMP-PNP, 1 mM	20	3.0	17	2
	GTP, 1 mM	26	4.8	21	7

* No proteinase K, Fig. 10, odd lanes *3–13*.
‡ After proteinase K, Fig. 10, even lanes *4–14*.
§ Difference of preceding two columns.
‖ At the end of the import assay.
¶ The concentration of [^{35}S]AOx was 86% of that before Sephadex; ATP was undetectable (<<1 μM).
** 1 mM ATP, 6 mM creatine phosphate, 5 μg/100 μl creatine phosphokinase.

(5, 8, 28) or out of bacteria (14, 43) where both ATP and a membrane potential are necessary (but see reference 4). Since GTP will not substitute for ATP, peroxisomal import again resembles chloroplast and yeast microsomal posttranslational import but differs from mitochondrial translocation.

The exact role of ATP in AOx import into peroxisomes is not yet known, but it is clear from our results that it functions in translocation, not in binding to the organelle. ATP could be involved in protein unfolding (34) and/or it could serve the membrane's putative translocation machinery. Skorin et al. have recently found (37) a 63-kD phosphoprotein and perhaps other phosphoproteins in the membrane of rat liver peroxisomes, which have unknown functions. One possibility, open to further study, is that ATP might drive import by a cycle of dephosphorylation and rephosphorylation of one of these membrane proteins.

The authors thank Mr. Gary Richo for excellent technical assistance, Ms. Helen Shio for the electron microscopy, Dr. Dean Cuebas for help with the experiment of Fig. 7, and Dr. Linda Randall (Washington State University) for the gift of SF6847.

This research was supported by National Institutes of Health grant AM 19394.

Received for publication 3 August 1987, and in revised form 8 September 1987.

References

1. Alexson, S. E. H., Y. Fujiki, H. Shio, and P. B. Lazarow. 1985. Partial disassembly of peroxisomes. *J. Cell Biol.* 101:294–304.
2. Bellion, E., and J. M. Goodman. 1987. Proton ionophores prevent assembly of a peroxisomal protein. *Cell.* 48:165–173.
3. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
4. Chen, L., and P. C. Tai. 1985. ATP is essential for protein translocation into *Escherichia coli* membrane vesicles. *Proc. Natl. Acad. Sci. USA.* 82:4384–4388.
5. Chen, W.-J., and M. G. Douglas. 1987. Phosphodiester bond cleavage outside mitochondria is required for the completion of protein import into the mitochondrial matrix. *Cell.* 49:651–658.
6. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.
7. de Duve, C., and P. Baudhuin. 1966. Peroxisomes (microbodies and related particles). *Physiol. Rev.* 46:323–357.
8. Eilers, M., W. Oppliger, and G. Schatz. 1987. Both ATP and an energized inner membrane are required to import a purified precursor protein into mitochondria. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1073–1077.
9. Findlay, J., G. A. Levvy, and C. A. Marsh. 1958. Inhibition of glycosidases by aldonolactones of corresponding configuration. 2. Inhibitors of β-N-acetylglucosaminidase. *Biochem. J.* 69:467–476.
10. Flugge, U. I., and G. Hinz. 1986. Energy dependence of protein translocation into chloroplasts. *Eur. J. Biochem.* 160:563–570.
11. Fujiki, Y., and P. B. Lazarow. 1985. Post-translational import of fatty acyl-CoA oxidase and catalase into peroxisomes of rat liver *in vitro*. *J. Biol. Chem.* 260:5603–5609.
12. Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment. Application to endoplasmic reticulum. *J. Cell Biol.* 93:97–102.
13. Fujiki, Y., R. A. Rachubinski, R. M. Mortensen, and P. B. Lazarow. 1985. Synthesis of 3-ketoacyl-CoA thiolase of rat liver peroxisomes on free polyribosomes as a larger precursor. Induction of thiolase mRNA activity by clofibrate. *Biochem. J.* 226:697–704.
14. Geller, B. L., N. R. Movva, and W. Wickner. 1986. Both ATP and the electrochemical potential are required for optimal assembly of pro-OmpA into *Escherichia coli* inner membrane vesicles. *Proc. Natl. Acad. Sci. USA.* 83:4219–4222.
15. Grossman, A., S. Bartlett, and N.-H. Chua. 1980. Energy-dependent uptake of cytoplasmically synthesized polypeptides by chloroplasts. *Nature (Lond.).* 285:625–628.
16. Hansen, W., P. D. Garcia, and P. Walter. 1986. In vitro protein translocation across the yeast endoplasmic reticulum: ATP-dependent posttranslational translocation of the prepro-α-factor. *Cell.* 45:397–406.
17. Hess, R., W. Staubli, and W. Riess. 1965. Nature of the hepatomegalic effect produced by ethylchlorophenoxy-isobutyrate in the rat. *Nature (Lond.).* 208:856–858.
18. Labarca, P., D. Wolff, U. Soto, C. Necochea, and F. Leighton. 1986. Large cation-selective pores from rat liver peroxisomal membranes incorporated to planar lipid bilayers. *J. Membr. Biol.* 94:285–291.
19. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of 3H and 14C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.
20. Lazarow, P. B., and C. de Duve. 1976. A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. *Proc. Natl. Acad. Sci. USA.* 73:2043–2046.
21. Lazarow, P. B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489–530.
22. Lazarow, P. B., Y. Fujiki, R. Mortensen, and T. Hashimoto. 1982. Identification of β-oxidation enzymes among peroxisomal polypeptides: increase in Coomassie Blue-stainable protein after clofibrate treatment. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 150:307–310.
23. Leighton, F., B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler, and C. de Duve. 1968. The large scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. *J. Cell Biol.* 37:482–513.
24. Mortensen, R. M. 1983. Clofibrate-induced increases in peroxisomal proteins: effects on synthesis, degradation, and mRNA activity. Ph.D. thesis. The Rockefeller University, New York. 67–84.
25. Mueckler, M., and H. F. Lodish. 1986. Post-translational insertion of a fragment of the glucose transporter into microsomes requires phosphoanhydride bond cleavage. *Nature (Lond.).* 322:549–552.
26. Pain, D., and G. Blobel. 1987. Protein import into chloroplasts requires a chloroplast ATPase. *Proc. Natl. Acad. Sci. USA.* 84:3288–3292.
27. Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247–256.
28. Pfanner, N., and W. Neupert. 1986. Transport of F_1-ATPase subunit β into mitochondria depends on both a membrane potential and nucleoside triphosphates. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 209:152–156.
29. Pfanner, N., and W. Neupert. 1987. Distinct steps in the import of ADP/ATP carrier into mitochondria. *J. Biol. Chem.* 262:7528–7536.
30. Rachubinski, R. A., Y. Fujiki, R. M. Mortensen, and P. B. Lazarow. 1984. Acyl-CoA oxidase and hydratase-dehydrogenase, two enzymes of the peroxisomal β-oxidation system, are synthesized on free polysomes of clofibrate-treated rat liver. *J. Cell Biol.* 99:2241–2246.
31. Reddy, J. K., S. K. Goel, M. R. Nemali, J. J. Carrino, T. G. Laffler, M. K. Reddy, S. J. Sperbeck, T. Osumi, T. Hashimoto, N. D. Lalwani, and M. Sambasiva Rao. 1986. Transcription regulation of peroxisomal fatty acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase in rat liver by peroxisome proliferators. *Proc. Natl. Acad. Sci. USA.* 83:1747–1751.
32. Reddy, J. K., J. R. Warren, M. K. Reddy, and N. D. Lalwani. 1982. Hepatic and renal effects of peroxisome proliferators: biological implications. *Ann. NY Acad. Sci.* 386:81–110.
33. Rothblatt, J. A., and D. I. Meyer. 1986. Secretion in yeast: translocation and glycosylation of prepro-α-factor in vitro can occur via an ATP-dependent post-translational mechanism. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1031–1036.
34. Rothman, J. E., and R. D. Kornberg. 1986. An unfolding story of protein translocation. *Nature (Lond.).* 322:209–210.
35. Schindler, C., R. Hracky, and J. Soll. 1987. Protein transport in chloroplasts: ATP is prerequisite. *Z. Naturforsch. Sect. C Biosci.* 41c:159–163.
36. Shore, G. C., and J. R. Tata. 1977. Two fractions of rough endoplasmic reticulum from rat liver. II. Cytoplasmic messenger RNA's which code for albumin and mitochondrial proteins are distributed differently between the two fractions. *J. Cell Biol.* 72:726–743.
37. Skorin, C., U. Soto, C. Necochea, and F. Leighton. 1986. Protein phosphorylation in peroxisomes. *Biochem. Biophys. Res. Commun.* 140: 188–194.
38. Small, G. M., and P. B. Lazarow. 1987. Import of the carboxyterminal portion of acyl-CoA oxidase into peroxisomes of *Candida tropicalis*. *J. Cell Biol.* 105:247–250.
39. Van Veldhoven, P., L. J. Debeer, and G. P. Mannaerts. 1983. Water- and solute-accessible spaces of purified peroxisomes. *Biochem. J.* 210: 685–693.
40. Van Veldhoven, P. P., W. W. Just, and G. P. Mannaerts. 1987. Permeability of the peroxisomal membrane to cofactors of β-oxidation. Evidence for the presence of a pore-forming protein. *J. Biol. Chem.* 262:4310–4318.
41. Verner, K., and G. Schatz. 1987. Import of an incompletely folded precursor protein into isolated mitochondria requires an energized inner membrane, but no added ATP. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2449–2456.
42. Waters, M. G., and G. Blobel. 1986. Secretory protein translocation in a yeast cell-free system can occur posttranslationally and requires ATP hydrolysis. *J. Cell Biol.* 102:1543–1550.
43. Yamane, K., S. Ichihara, and S. Mizushima. 1987. In vitro translocation of protein across *Escherichia coli* membrane vesicles requires both the proton motive force and ATP. *J. Biol. Chem.* 262:2358–2362.