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ATPase activities in peroxisome-proliferating yeast

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Preliminary studies on yeast peroxisomes have suggested that the membrane of these organelles may contain a proton-pumping ATPase. It has been reported that peroxisome-associated activity is similar to the F_0-F_1 mitochondrial type ATPase in its sensitivity to azide at pH 9.0, but characteristics of the plasma membrane type ATPase are also evident in peroxisomal preparations in that they exhibit pH 6.5 activity that is sensitive to vanadate. A comparative study of the prominent organellar ATPase activities was undertaken as a probe into the existence of an enzyme that is unique to the peroxisome, and biochemical properties of yeast mitochondrial, plasma membrane, together with peroxisomally-associated H^+-ATPases are presented. Enzyme marker analysis of sucrose gradient fractions revealed a high degree of correlation between the amount of azide-sensitive pH 9.0 ATPase activity and that of the mitochondrial membrane marker, cytochrome *c* oxidase, in peroxisomal preparations. Purified mitochondrial and peroxisomally-associated activities were highly sensitive to the presence of sodium azide, *N,N'*-dicyclohexylcarbodiimide (DCCD) and venturicidin when measured at pH 9.0. Comparisons of peroxisomal activities with those of the purified plasma membrane at pH 6.0 in the presence of azide showed similar sensitivity profiles with respect to inhibitors of yeast plasma membrane ATPases such as vanadate and *p*-chloromercuriphenyl-sulfonic acid (CMP). Purified peroxisomal membranes, furthermore, reacted with antibody to the mitochondrial F_1 subunit (as revealed by Western blot analysis), and [^{35}S]methionine-labeled, glucose-grown cells processed with unlabeled methanol-grown cells, yielded sucrose gradient fractions that were radioactive in bands that were also recognized by F_1 antibody. Isolated fractions in these experiments had similar ratios of cpm : pH 9.0 ATPase activities, suggesting that this activity is mitochondrial in origin. The data presented for the characteristics of the peroxisomally-associated activity strongly suggest that the majority of the ATPase activity found in peroxisomal preparations is derived from other organelles.

Introduction

Peroxisomes from methanol-grown *Candida boidinii* house the initial enzymes of the methanol assimilation pathway, namely alcohol oxidase, dihydroxyacetone synthase and catalase. One of these enzymes, alcohol oxidase, has been shown to assemble posttranslationally into the organelle as a crystalline homo-octamer [1] and the mechanism of this assembly is currently an area of active research. It has been shown, for instance, that proton ionophores prevent the octamerization of alcohol oxidase in activated spheroplasts and that removal of these agents leads to normal assembly [2]. In light of these findings, it has been suggested that an energized peroxisomal membrane may exist that facilitates the assembly of alcohol oxidase subunits and that this potential is nullified in the presence of ionophores.

Initial investigations of ATPase activity associated with purified peroxisomal membranes have been reported by us and others [47,48]. Douma et al. [3] have reported that a mitochondrial-like ATPase is found associated with the peroxisomal membrane of *Hansenula polymorpha* when the organism is grown on methanol, and that ATP-dependent Ce^{3+}-ion staining of the membrane further substantiates the existence of an ATPase on the surface of the organelle. Later work revealed that peroxisomal preparations contained material that cross-reacted with antibody against mitochondrial ATPase from yeast [4] although other evidence indicated that the preparations were not contaminated by mitochondria. Two reports have docu-

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Abbreviations: SDS, sodium dodecyl sulfate; DCCD, *N,N'*-dicyclohexylcarbodiimide; CMP, *p*-chloromercuriphenylsulfonic acid.

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mented the possible presence of an ATPase in rat liver peroxisomes. One of these [5] suggested that since the activity was not inhibited by oligomycin, the enzyme was of the lysosomal/vacuolar type. The other report [6] which, in contrast, described work done using highly purified peroxisomal preparations, indicated that although there were contaminating ATPases present, these were not sufficient to account for all of the observed activity. In addition, they also showed cross-reactivity with antibody against rat liver mitochondrial ATPase β subunit. Our initial findings had indicated the presence of ATPase activity associated with peroxisomal membrane preparations from *Candida boidinii*. This activity showed, by several criteria, similarity to ATPases of both the mitochondrial and plasma membrane types, but not the vacuolar type. In order to resolve the nature of this activity we undertook a comparative study of H^+-ATPases in this organism as a probe into the possible existence of a unique peroxisomal H^+-ATPase. (A preliminary account of this work was presented at the I.U.B. 14th International Congress of Biochemistry, Prague, Czechoslovakia, 1988.)

Mitochondrial, plasma membrane and vacuolar H^+-ATPases are common to eucaryotic cells and are distinguishable by such biochemical characteristics as pH optima, substrate specificities and sensitivities to various inhibitors. The mitochondrial enzyme, for instance, is made up of a catalytic portion (F_1) composed of five different size subunits designated in descending order of molecular weight α–ϵ. The catalytic portion of the enzyme complex is attached to an integral membrane-bound portion (F_0) which is composed of multiple hydrophobic subunits [7]. The F_0-F_1 enzyme is highly sensitive to azide, oligomycin and venturicidin, has maximal activity at pH values between 8.2 [8] and 9.0–9.5 [9,10], and has an estimated K_m for Mg^{2+}-ATP of 0.3 to 0.8 mM [11]. Evidence for the presence of another type of H^+-ATPase in the plasma membrane of yeasts has been obtained for several different species, including *Saccharomyces cerevisiae* [12–17], *Candida tropicalis* [18–20], *Candida albicans* [21], *Neurospora crassa* [22–24] and *Schizosaccharomyces pombe* [25,26]. This enzyme has one large subunit of 105 kDa, which becomes phosphorylated at a specific aspartyl residue during the reaction [28,29]. It is highly sensitive to inhibition by vanadate [14–17,20] but is not inhibited by azide nor by oligomycin [12,13,15–17,20,25]. The pH optimum of the plasma membrane ATPase derived from *S. cerevisiae* is in the region of 5.5–5.6 [15–17], that from *S. pombe* is 6.0 [25], while those of *C. albicans* and *N. crassa* are reported to be 6.5, 6.4 and 6.7, respectively [20,21,24]. *C. tropicalis* plasma membrane ATPase has a pH optimum of 6.5–7.0 when assayed from glucose-grown cells [18,20] but this value is 8.0 in hexadecane-grown cells [18]. The yeast vacuolar enzyme is unique in that it is insensitive to all the inhibitors mentioned above, but is very sensitive to *N,N'*-dicyclohexylcarbodiimide (DCCD) [30,31,33]. Vacuolar ATPases have neutral pH optima (around 7.5) and a K_m for ATP, 0.2 mM, close to that of the mitochondrial ATPase [31–34]. The plasma membrane enzyme is highly specific for ATP, hydrolyzing other nucleotides at a rate less than 7% of the activity with ATP [15,16,20,21,25,32], whereas mitochondrial and vacuolar ATPases will also hydrolyze GTP, ITP, UTP and CTP [11,15,25,31–33]. Distinctions of these types have been used routinely to judge the purity of an organellar preparation [10,30,35] and have been utilized in the current study to determine the source of the activity found associated with highly purified peroxisomal membranes.

Materials and Methods

Membrane preparations. Cells of *C. boidinii*, grown on 1% methanol plus salts medium, were harvested and peroxisomal membranes isolated by differential and sucrose gradient centrifugations as described by Goodman et al. [36] except that the initial sucrose gradients contained 14 ml each of 35 and 50% sucrose (w/w) layered over a cushion of 2 ml 60%. Gradient fractions for enzyme marker analysis were collected as the whole of material isolated from the middle of one sucrose layer to the middle of the next. Fractions were diluted with 2–3 volumes of 10 mM Tris (pH 8.0), and membranous material was pelleted by centrifugation at $118\,000 \times g$ for 1 h. Material migrating to the 50/60% interface of these gradients contained intact peroxisomes that could be collected into centrifuge tubes and lysed by the addition of 30 mM Tris-HCl (pH 8.0). Peroxisomal membranes were floated in a second gradient by overlayering the lysed organelles with 10 ml each 50 and 40% sucrose, filling to volume with 30% sucrose and centrifuging at $19\,900 \times g_{avg}$ for 36–42 h. The sucrose solutions for the second gradient contained 5 mM Tris-HCl (pH 8.0). Fractions from this second gradient were also collected from the top of the tube, and are designated 30M, 40M and 50M in order to distinguish these fractions from those of the first gradient. Floated membranes were resuspended in 10 mM Tris-HCl (pH 8.0) containing 5 μg/ml each aprotinin, leupeptin, *N*-tosyl-L-phenylalanine chloromethylketone, *N*-α-*p*-tosyl-L-lysine chloromethylketone and 1 mM phenylmethylsulfonyl fluoride.

Mitochondria were isolated from glucose-grown *C. boidinii*. Discontinuous sucrose gradients were constructed similar to those for peroxisomal preparations except for the inclusion of 40 and 45% sucrose layers. The 35/40% interface material was substantially free of peroxisomes as indicated by the absence of a 70 kDa (alcohol oxidase) band in SDS-PAGE, and had a high specific activity of the mitochondrial type ATPase.

Material from this layer was subsequently washed and reconstituted in 10 mM Tris-HCl (pH 8.0), containing 1 mM EDTA and proteinase inhibitor mix as above.

Plasma membranes were isolated and purified also from glucose-grown *C. boidinii* according to the methods of Bowman et al. [21,37].

Enzyme and protein assays. H^+-ATPase was routinely assayed as the amount of inorganic phosphate released in buffered solutions containing 5 mM each ATP and $MgCl_2$ at 30°C. Reactions were stopped after 5–60 min and phosphate concentrations were measured by methods described by Ames [38]. Phosphate was produced as a linear function of time and enzyme concentration. Cytochrome *c* oxidase (EC 1.9.3.1) activity was measured by the standard technique of Tolbert [39]. Alcohol oxidase (EC 1.1.3.13) was assayed as previously described by Goodman et al. [1] except that the reaction was stopped by the addition of trichloroacetic acid such that the final concentration was 0.2% (v/v). For enzyme marker analysis of fractions collected throughout peroxisomal purifications, mitochondrial ATPase was assessed as the amount of azide-sensitive pH 9.0 activity, and plasma membrane activity was determined at pH 6.0 with and without the addition of 100 mM vanadate. Purified plasma membranes were assayed in the presence of 5 mM azide in order to inhibit residual mitochondrial activity, whereas purified mitochondrial activities were unaffected by the presence of 100 mM vanadate and were assayed in the absence of inhibitor.

Protein concentrations were initially estimated by the Bradford method [40]. More accurate assessment of membrane protein concentration was obtained by Amido black staining as described by Schaffner and Weissman [41] following solubilization of membrane protein in 0.24% SDS.

Determination of pH optima. ATPase activities were assayed as previously described under 'Enzyme and protein assays' except for the variation of buffer solutions. Stock solutions of Mes were adjusted in the pH range of 5.5 to 6.5 with uncorrected Tris base. Hepes and Ches were used for higher pH values (7.0 to 8.0 and 8.5 to 9.5, respectively) and likewise adjusted with Tris.

Inhibitor sensitivity assays. ATPase activities were assayed as described with the addition of inhibitors in varying concentrations. Sodium azide, vanadate and *p*-chloromercuriphenylsulfonic acid (CMP) were directly added to assay mixtures from aqueous stock solutions, whereas venturicidin and DCCD were dissolved in and diluted with 95% ethanol. Final ethanol concentrations were less than 0.5% in the assay mixtures and control experiments showed negligible enzyme inhibition by ethanol alone at these concentrations.

Kinetic studies. Assays for the kinetic properties of ATPases were as described for routine assays except that the reactions were initiated by the addition of Mg^{2+}-ATP-Tris solutions in the range of 5 to 100 mM for final ATP concentrations between 0.5 and 10 mM. Hanes plots of [ATP]/initial rate versus [ATP] were used to estimate K_m values.

Electrophoresis and Western blots. Sucrose gradient fractions were electrophoresed on 9% gels as described by Goodman et al. [1]. Purified mitochondrial F_1 subunit was similarly electrophoresed and the proteins were subsequently electrotransferred onto nitrocellulose by Western blot techniques [42]. Blotted proteins were incubated with antibody to F_1 proteins which were subsequently visualized by incubations in peroxidase-conjugated goat anti-rabbit IgG in the presence of hydrogen peroxide and 4-chloro-1-naphthol.

Mitochondrial labeling experiments. C. boidinii were grown overnight on 2% glucose in order to repress the production of peroxisomes. [^{35}S]Methionine was added to these incubations at the time of inoculation (300 mCi/2 l culture) in order to produce general protein labeling. Peroxisomes were induced in separate cultures of methanol-grown *C. boidinii* as described earlier. Labeled and unlabeled cells were collected and washed separately, but were combined for conversions to spheroplasts and further processed as a standard peroxisomal preparation. Sucrose gradient fractions were collected from the 35/50 and 50/60% interfaces of the initial sucrose gradient, but the bulk of material at the peroxisomal interface was floated in a second gradient as described earlier. Each fraction was subsequently washed and resuspended in 10 mM Tris-HCl (pH 8.0) containing proteinase inhibitors. Fractions were analyzed by Western blot techniques using antiserum to yeast mitochondrial F_1 subunit, and the resulting blots were also autoradiographed. ATPase activities were assessed, and samples of the material were counted by liquid scintillation.

Reagents. Adenine nucleotides, *p*-chloromercuriphenylsulfonic acid (CMP), venturicidin, sodium orthovanadate and proteinase inhibitors were all purchased from Sigma. All other nucleotides were purchased from U.S. Biochemical Corporation, DCCD was from Aldrich and sodium azide was from J.T. Baker. ^{35}S-Labeled methionine was obtained from Dupont/New England Nuclear Research Products and the purified yeast mitochondrial F_1 subunit, as well as the antibody to it, were the kind donation of Prof. M. Douglas of the University of Texas Health Science Center at Dallas.

Results

Enzyme marker analysis

Purity of the peroxisomal membrane preparation from methanol-grown cells was evaluated on the basis

of pelletable marker enzyme activity. Total activities of the enzymes found associated with washed sucrose gradient fractions are presented in Table I. 96 percent of the cytochrome *c* oxidase activity, for instance, was collected from the 35/50% interface of the first sucrose gradient, and represents the localization of mitochondria to this layer. Likewise, 96 percent of the total azide-sensitive H^+-ATPase activity (pH 9.0) migrated to the 35/50% sucrose interface, and is therefore likely to be the mitochondrial F_0-F_1 enzyme. Less than 1% of both the cytochrome *c* oxidase and the azide-sensitive ATPase activity was recovered from the peroxisomal 50/60% sucrose layer and, as such, indicates the degree of peroxisomal contamination by mitochondria. Only 15% of the total azide-sensitive activity associated with peroxisomes survived further purification of the membranes on the second sucrose gradient, where 24% of the total gradient cytochrome *c* oxidase activity is also found. The specific activity of both of these marker enzymes decreases 2–2.5-fold as a result of peroxisomal membrane flotation in the second gradient. The plasma membrane marker enzyme is the vanadate-sensitive ATPase activity measured at pH 6.5 and the bulk of this activity (94 percent) was found associated with the 35/50% mitochondrial fraction. Only 3% of the total activity that was isolated from this gradient was found in the 50/60% peroxisomal fraction, and only 6% of this was found associated with purified peroxisomal membranes. A 6.5-fold decrease in the specific activity of this marker occurred during peroxisomal membrane purification.

There are currently no known enzymes unique to peroxisomal membranes; this fact presents an obstacle to the convenient assay for the presence of these membranes. However, alcohol oxidase, a matrix enzyme, is also usually found in association with peroxisomal membranes, and is removed from them only by high pH wash [36]. It may therefore be used as a useful marker for the peroxisomal membrane. Membrane-associated alcohol oxidase activity partitioned to the 50/60% interface of the first gradient, and 35% of membrane-associated activity co-purified with peroxisomal membranes (an increase in specific activity from 1.9 to 2.4 U/mg). The apparently low recovery of alcohol oxidase in these preparations arises because most of the enzyme remains in the supernatant fluid following lysis of whole peroxisomes and the assays were done on pelleted membrane samples. In terms of total activity more than 80% of applied alcohol oxidase was found at the 50/60% interface. The high alcohol oxidase activity found associated with mitochondria (35/50% interface) is thought to be due to incomplete separation of peroxisomes (or their cores) from mitochondria in this preparation.

Gel electrophoresis

The protein profiles of purified peroxisomal membranes are shown in Fig. 1 alongside those of mitochondria and plasma membranes isolated by means of separate procedures. Characteristic protein bands for the mitochondrial and plasma membrane ATPases are indicated.

pH optima

H^+-ATPase activity associated with the floated peroxisomal membrane exhibited two pH maxima (6.5 and 9.0) when assayed in the absence of inhibitors (Fig. 2). The activity at pH 6.5 was inhibited 44% by 100 mM vanadate where the activity at pH 9.0 was inhibited by 18%. Conversely, the pH 9.0 activity decreased by 90% in the presence of 5 mM sodium azide, whereas the pH 6.5 activity was diminished by 52% and very little activity remained at any pH when assayed in the presence of both inhibitors. The pH profiles of the isolated

TABLE I

Total enzyme activity [a] found associated with membranes collected from sucrose gradients

	Protein (mg)	Azide-sens. pH 9.0 ATPase	Van.-sens. pH 6.5 ATPase	AO	Cytochrome *c* oxidase	Ratio 9.0/Cyt. [b]
Top	2.5 (7) [c]	2.5 (4)	0.35 (3)	1.2 (9)	170 (4)	0.015
35/50	29 (83)	61 (96)	11 (94)	6.0 (44)	4330 (96)	0.014
50/60	3.3 (9)	0.25 (0.4)	0.30 (3)	6.4 (47)	16.4 (0.4)	0.015
% Recovered [d]	82	339	128	24	179	
50M	1.6 (24)	0.19 (30)	0.26 (49)	2.7 (17)	3.98 (17)	0.048
40M	2.8 (41)	0.34 (54)	0.24 (45)	7.8 (48)	14.1 (60)	0.024
30M	2.4 (35)	0.097 (15)	0.034 (6)	5.7 (35)	5.57 (24)	0.017
% Recovered	69	84	59	84	34	

[a] Activity expressed as μmol/min except for oxidase activity which is expressed as ΔA_{415nm}/min.
[b] Ratio of pH 9.0 ATPase activity relative to the activity of cytochrome *c* oxidase.
[c] Numbers in parentheses represent the percent of total activity collected from each gradient.
[d] Percent of total activity recovered in gradient.

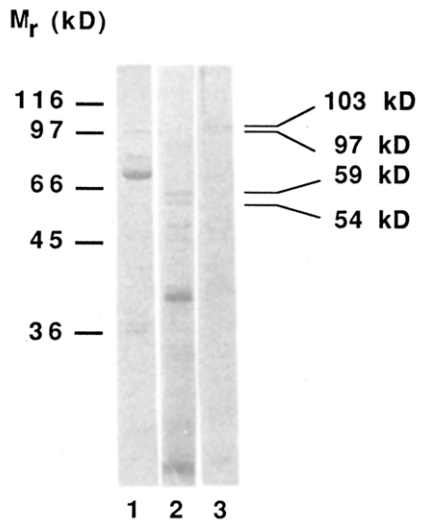

Fig. 1. SDS-PAGE of floated peroxisomal membranes, mitochondrial membranes and plasma membrane ATPase isolated by procedures described under Materials and Methods. Lanes contain: (1) 30M peroxisomal membrane fraction, 10 μg membrane protein; (2) 30/50% sucrose gradient fraction (mitochondrial), 13.5 μg membrane protein; (3) plasma membrane ATPase, 100KS2 deoxycholate-solubilized fraction, 10 μg membrane protein. Positions of molecular mass markers are shown on the left; molecular mass labels on the right side refer to prominent bands in the preparations, i.e., 103 kDa and 97 kDa refer to bands in lane 3, whereas 59 kDa and 54 kDa refer to bands in lane 2.

mitochondrial and plasma membranes are shown in Fig. 3 with their corresponding maxima at 9.0–9.5 and 6.0 (6.5 without azide), respectively.

Inhibitor sensitivities

Comparative investigations of membrane sensitivities to inhibitors are illustrated in Fig. 4. Varying the azide concentration from 0.25 to 5 mM in the plasma membrane incubations inhibits the liberation of inorganic phosphate by 5 to 24% with a further decline to 55% of the original activity at concentrations greater than 25 mM (Fig. 4a). Peroxisomal membrane preparations parallel the sensitivities of mitochondrial ATPase to azide with $[I]_{0.5}$ of 0.3 and 0.2 mM, respectively, suggesting that the pH 9.0 activity associated with peroxisomes is very similar to the mitochondrial-type enzyme. When assayed in the presence of vanadate, peroxisomal pH 9.0 activity is further sensitized to azide, and the profile of inhibition becomes essentially coincident with the mitochondrial profile (data not shown). Similar trends were found with varying concentrations of other inhibitors of the mitochondrial type ATPase such as venturicidin and DCCD (Fig. 4c and d) where the ATPase activity found associated with purified peroxisomal membranes paralleled the sensitivity of either the mitochondrial or plasma membrane type activities when assayed at the corresponding pH in the presence of either vanadate (not shown) or azide.

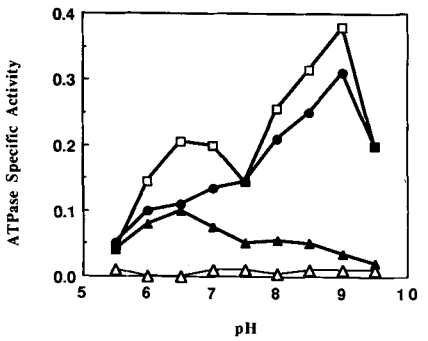

Fig. 2. Effect of pH on peroxisomal membrane-associated ATPase activity (μmol P_i min^{-1} mg $protein^{-1}$) assayed as described under Materials and Methods in the absence of inhibitors (□). Similar pH profiles are also presented for activities in the presence of 100 μM vanadate (●), as well as activities in the presence of 5 mM azide (▲) and in the presence of both inhibitors (△).

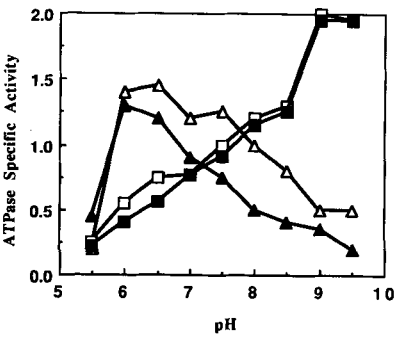

Fig. 3. Effect of pH on purified mitochondrial and plasma membrane fractions assayed in the presence and absence of 100 μM sodium vanadate. Plasma membrane ATPases were assayed similarly but in the presence and absence of 5 mM sodium azide. Assays were performed as described under Materials and Methods and specific activities are presented as μmol P_i min^{-1} mg $protein^{-1}$. Symbols: □ mitochondria; ■ mitochondria plus 100 μM sodium vanadate; △ plasma membranes; ▲ plasma membranes plus 5 mM sodium azide.

Comparisons of the sensitivities of the three membrane preparations to inhibitors of the plasma membrane type ATPase are shown in Fig. 4b and e. Vanadate does not significantly inhibit mitochondrial activity, whereas plasma membranes are inhibited 98% by 100 mM vanadate (Fig. 4b). Peroxisomal membranes approach the degree of sensitivity of the plasma membranes to this inhibitor at concentrations above 10 mM when assayed at pH 6.0 in the presence of azide, whereas pH 9.0 peroxisomal activity only becomes significantly inhibited at vanadate concentrations in excess of 100 mM. Sensitivity of the peroxisomal activity to CMP was also pH dependent (Fig. 4e) and demonstrated the characteristic shift in sensitivity to this inhibitor towards that of either the plasma membrane or mitochondrial ATPase when assayed under similar conditions.

Substrate affinity

The apparent K_m for Mg^{2+}-ATP was also assessed for all three membrane preparations. Mitochondrial

preparations measured at pH 9.0 had an apparent K_m of 0.35 mM (Fig. 5), where that of the purified plasma membrane ATPase was 1.4 mM when assayed at pH 6.0 in the presence of 5 mM azide (Fig. 6). Hanes plots for the peroxisomal activity at pH 9.0 with and without 100 μM vanadate are also shown in Fig. 5 with corre-

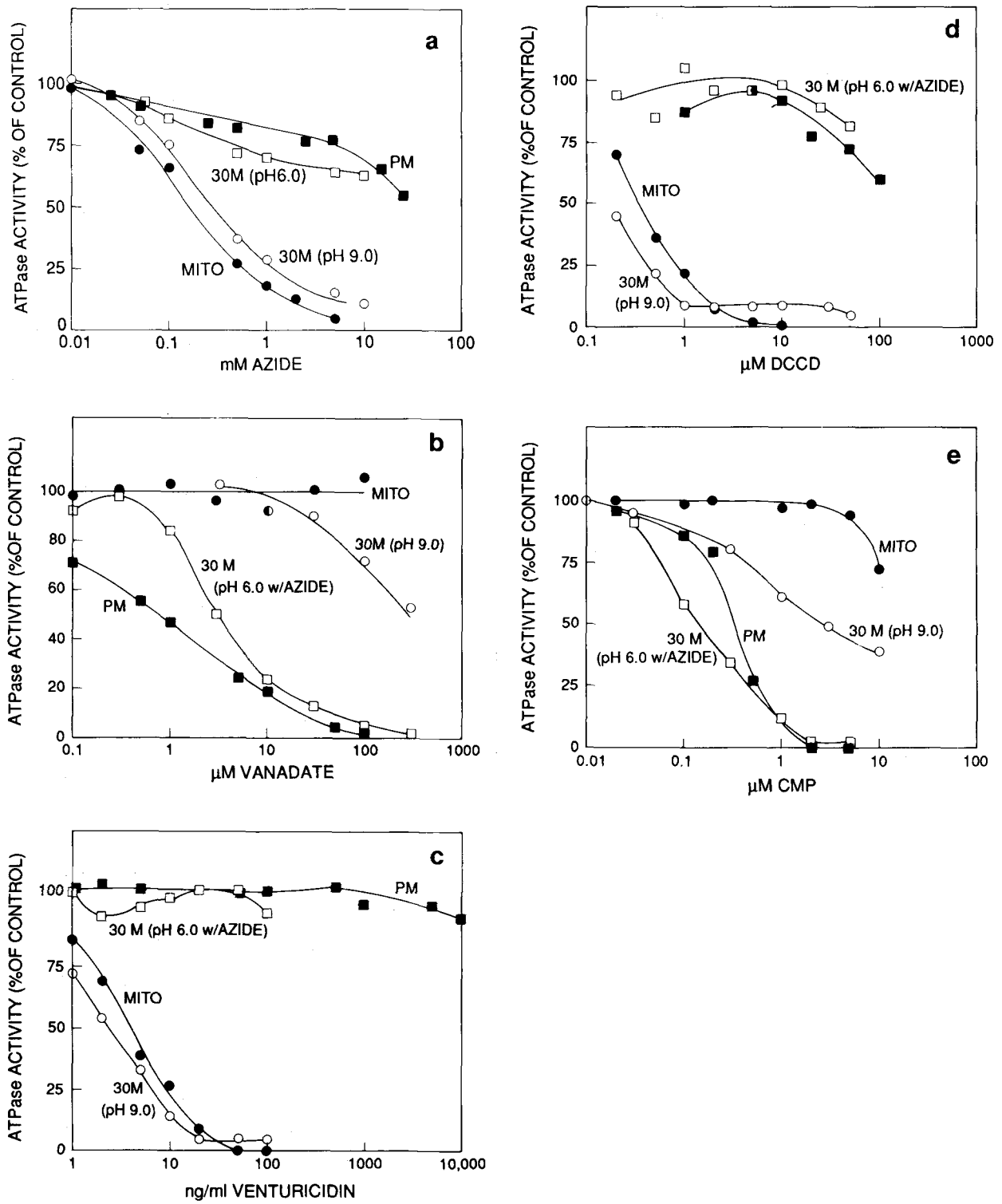

Fig. 4. Inhibitor sensitivities of mitochondrial (closed circles), plasma membrane (closed squares) and purified peroxisomal ATPases assayed at pH 9.0 (open circles) and at pH 6.0 in the presence of 5 mM azide (open squares). Relative activities are presented as the percent of control. Inhibitors were added from concentrated stock solutions as described under Materials and Methods with the indicated final concentrations of (a) azide, (b) vanadate, (c) venturicidin, (d) DCCD and (e) CMP. Mitochondria and plasma membranes were assayed as described under Materials and Methods except that plasma membrane sensitivity to azide (a) was assayed in the absence of mitochondrial inhibitor. Peroxisomally-associated activity was assayed at pH 9.0, or at pH 6.0 in the presence of azide.

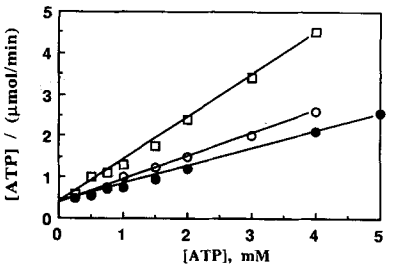

Fig. 5. Hanes plots for the mitochondrial ATPase measured at pH 9.0 (□) and that of the peroxisomal activity measured at pH 9.0 with (●) and without vanadate (○).

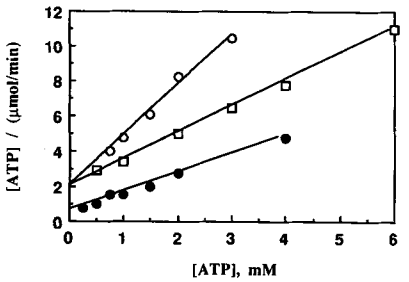

Fig. 6. Hanes plots for the plasma membrane ATPase measured at pH 6.0 in the presence of 5 mM azide (□) and that of the peroxisomal activity measured at pH 6.5 with (●) and without (○) azide.

sponding K_m of 0.76 and 0.67 mM, respectively. Likewise, the peroxisomal affinity for substrate was assessed at pH 6.5 with and without the addition of 5 mM azide and the estimated K_m values were 0.63 and 0.50 mM, respectively (Fig. 6). Kinetic data for the activity associated with peroxisomes presented here are essentially similar to those of the mitochondrial and plasma membrane enzymes, in contrast to a previous report [3], where the substrate affinity of the peroxisomal activity was 10-fold lower than that of the mitochondrial enzyme.

Substrate specificity

The specificity for substrate nucleotides was also investigated and the results are presented in Table II.

TABLE II

Nucleotide specificity of membrane preparations presented as specific activity (μmol P_i min^{-1} mg $protein^{-1}$)

Nucleotide (5 mM)	Mito	30M (pH 9.0)		PM	30M (pH 6.5)	
		−Van	+Van		−Az	+Az
ATP	2.55	0.186	0.209	1.12	0.126	0.023
GTP	1.16	0.080	0.093	n.d.	0.052	n.d.
ITP	1.05	0.110	0.101	n.d.	0.080	n.d.
UTP	0.54	0.035	0.020	n.d.	0.045	n.d.
CTP	n.d. [a]	n.d.	n.d.	n.d.	n.d.	n.d.
XTP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ADP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
AMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] None detected (n.d.) within the limits of sensitivity of the assay (< 0.02 μmol P_i min^{-1} mg $protein^{-1}$).

TABLE III

Divalent cation specificity for membrane preparations presented as specific activity (μmol P_i min^{-1} mg $protein^{-1}$)

Metal ion (5 mM)	Mito	30M (pH 9.0)		PM	30M (pH 6.5)	
		−Van	+Van		−Az	+Az
Mg^{2+}	2.19	0.205	0.231	0.514	0.140	0.028
Mn^{2+}	1.94	0.148	0.175	0.138	0.128	n.d.
Fe^{2+}	1.93	0.203	0.240	n.d.	0.050	n.d.
Co^{2+}	1.72	0.070	0.084	0.099	0.097	n.d.
Ca^{2+}	1.15	0.050	0.056	n.d.	n.d.	n.d.
Zn^{2+}	n.d. [a]	n.d.	n.d.	0.121	0.045	n.d.
Cu^{2+}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
none	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] None detected (n.d.) within the limits of sensitivity of the assay (< 0.02 μmol P_i min^{-1} mg $protein^{-1}$).

Plasma membranes showed nearly absolute specificity for Mg^{2+}-ATP. Mitochondrial activities, on the other hand, approach 50% of that obtained with ATP when substituted with equimolar GTP or ITP. UTP was also hydrolyzed by mitochondria, but only 21% as much as ATP. Peroxisomal membranes, similarly to mitochondrial and plasma membranes, hydrolyzed no detectable amounts of CTP, XTP, ADP or AMP at either pH.

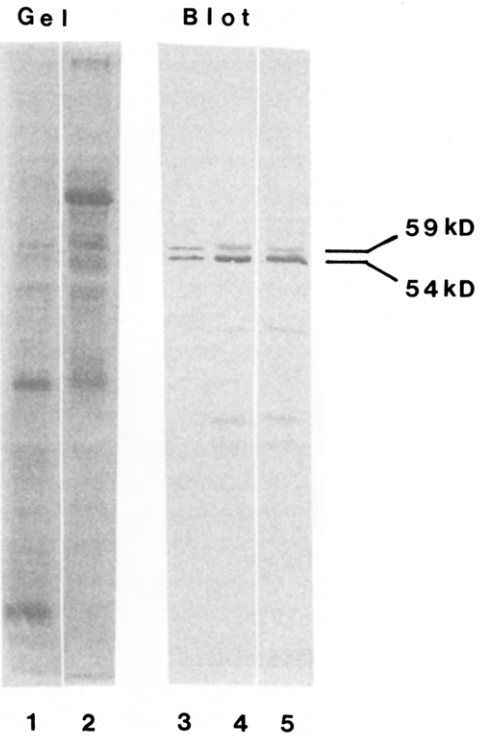

Fig. 7. Immunochemical analysis of mitochondrial and peroxisomal membrane preparations. Samples of mitochondrial preparations, containing 13 μg protein, (lanes 1 and 4) and peroxisomal membrane preparations, 22 μg protein, (lanes 2 and 5) were subjected to SDS-PAGE and stained with Coomassie blue (left panel). Western blots of these gels were probed with antibody against the F_1 ATPase fragment of *S. cerevisiae* (right panel). Lane 3 contained purified mitochondrial F_1 subunit (6 μg protein) from *S. cerevisiae*.

The peroxisomally-associated activity with GTP varied between 42 and 44 % of control when measured at pH 9.0 with or without vanadate or at pH 6.5 when no azide was present and resembles the behavior of the mitochondrial preparations. Peroxisomal activities measured at pH 6.5 in the presence of azide were below the limits of sensitivity when other nucleotides were substituted for ATP.

The specificity for divalent cations is depicted in Table III and shows the relative acceptability of Mn^{2+}, Fe^{2+}, Co^{2+} and (to a lesser degree) Ca^{2+} as substitutes for Mg^{2+} ion by mitochondrial membranes, whereas plasma membranes show little activity with these or other ions relative to Mg^{2+}. Activities found associated with peroxisomal membranes, as for those of the mitochondrial enzyme, were relatively insensitive to substitutions of Mg^{2+} by either Mn^{2+} or Fe^{2+} when measured at pH 9.0, whereas activities with Co^{2+} or Ca^{2+} are somewhat reduced. Peroxisomal activities measured at pH 6.5 are also relatively nonspecific for divalent cation. The addition of azide to the reaction mixture, however, reduces the total activity at this pH and substitutions for Mg^{2+} reduce activities below detectable limits.

Immunochemical analysis

Since the kinetic properties of the ATPase activity associated with peroxisomal membranes appeared to resemble closely those of mitochondrial ATPase, we decided to determine if mitochondrial ATPase was in fact present in these preparations. Western blots from SDS-PAGE gels used to resolve samples of floated peroxisomal membranes were probed for mitochondrial ATPase components by using antibody raised against F_1 ATPase from *S. cerevisiae*. The results (Fig. 7) show clearly that F_1 antibody recognizes proteins in the peroxisomal preparations (lane 5) in a similar fashion to those in mitochondrial preparations (lane 4).

Mitochondrial labeling

Co-migration of mitochondrial ATPase activity in peroxisomal preparations was confirmed by experiments in which labeled glucose-grown cells (essentially devoid of peroxisomes [43]) were processed with unlabeled methanol-grown cells in an otherwise unmodified peroxisomal preparation. Western blots of the fractions revealed the location of the F_1-immunodecorated bands (Fig. 8) and autoradiography of the blotted proteins demonstrated the extent of radioactivity at these locations. The prominent alcohol oxidase band in the purified peroxisomal membrane fraction, however, is not labeled and, as such, rules out the possibility that the peroxisomes might have been labeled in the course of processing.

Diluted fractions collected from the 35/50% sucrose interface allowed the comparison of equi-volume

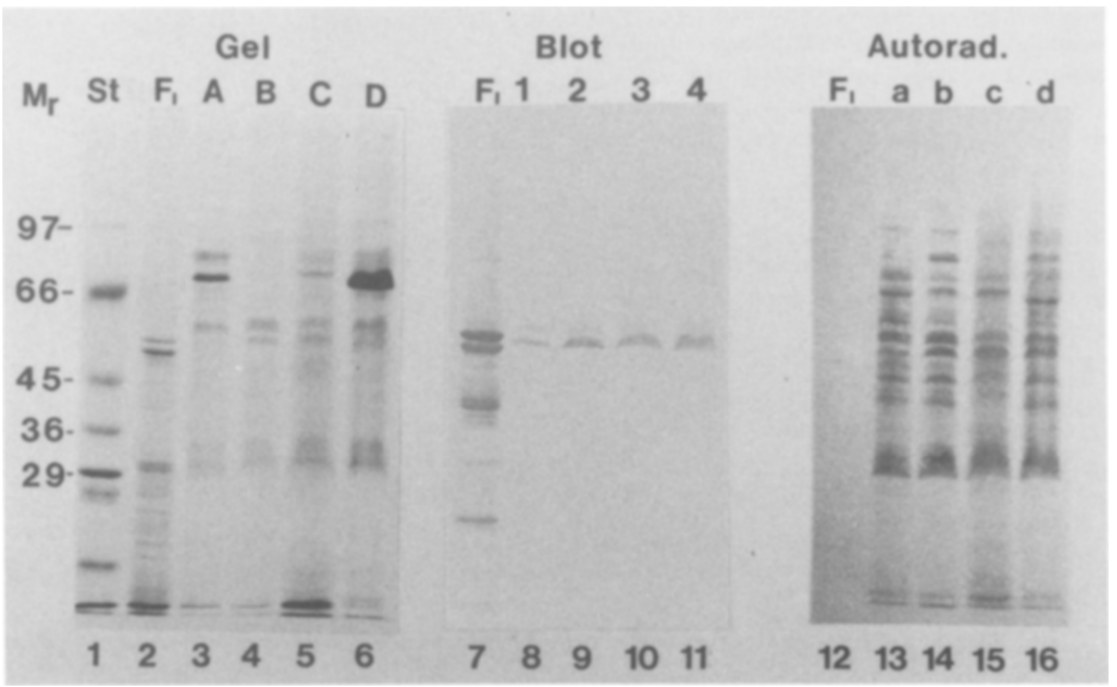

Fig. 8. Gel electrophoresis with Coomassie blue staining (left panel) and Western blot (probed with antibody against the F_1 ATPase fragment of *S. cerevisiae*, center panel) analysis of sucrose gradient fractions collected from peroxisomal preparations that were derived from a mixture of glucose-grown [^{35}S]methionine-labeled cells and methanol-grown unlabeled cells of *C. boidinii*. The right panel shows an autoradiogram of the blot membrane. Lanes were loaded with 15 μg membrane protein each of: 12K pellet fraction (A, 1, a), 35/50% fraction (B, 2, b), 50/60% fraction (C, 3, c) and the 30M fraction. F_1 indicates lane loaded with purified mitochondrial F_1 subunit (15 μg protein) from *S. cerevisiae*.

TABLE IV

Activity and labeling of sucrose gradient fractions after mixing labeled glucose-grown cells with unlabeled methanol-grown cells

Fraction	Protein (μg/10 μl)	cpm (10 μl)	Activity [a] (nmol/min)	Ratio cpm/activity
35/50	6.97	14800	7.08	1900
50/60	13.5	17800	5.82	2070

[a] Azide-sensitive ATPase activity measured at pH 9.0 for each 10 μl sample.

(10 ml) enzyme activity and radioactivity in this fraction to that of the 50/60% fraction (Table IV). The ratio of counts per min to total azide-sensitive ATPase activity in these aliquots was 1900 and 3070 for the mitochondrial and peroxisomal fragments, respectively, and further substantiates the suggestion that mitochondrial fragments are present in peroxisomal layers.

Discussion

The majority of ATPase activity observed during the purification of peroxisomal membranes was found in non-peroxisomal fractions (Table I). ATPase activity in general, therefore, did not appear to co-purify with peroxisomes. Investigation of the activity that does remain, however, revealed two pH maxima. The pH 6.5 activity was largely inhibited by 100 μM vanadate, and the activity at pH 9.0 was sensitive to azide. To date there is no known single enzyme that exhibits all of these characteristics. The presence of mitochondrial contamination was indicated by cytochrome *c* oxidase activity in floated, washed peroxisomal membranes. It is likely, therefore, that the pH 9.0 H^+-ATPase activity found associated with peroxisomes is also due to the presence of mitochondrial membranes, and the ratio of azide-sensitive ATPase to cytochrome *c* oxidase activities reveals the expected positive correlation (Table I). The higher proportion of ATPase activity relative to that of cytochrome *c* oxidase in fractions collected from the second gradient is more likely due to the loss of oxidase activity (35% recovery) over the extended centrifugation period (36–42 h) than it is a reflection of a relative increase in ATPase activity (84% recovery). This loss of cytochrome *c* oxidase activity over extended periods in concentrated sucrose solutions has been noted by others also [39], and after corrections for the inactivation of the oxidase to the degree of pH 9.0 ATPase recovery, the ratios become 0.020, 0.010, 0.007 for the 50M, 40M and 30M fractions, respectively.

The greater than 100% recovery of azide-sensitive ATPase in gradient fractions may be due to the removal or inactivation of endogenous inhibitors or fractionation of the mitochondrial enzyme itself. Release of a 'latent ATPase' [44], for instance, was one of the first descriptions of the dramatic increase in ATPase activity as the F_1 subunit was solubilized from the F_0-F_1 complex. Fragmentation of the holoenzyme in our preparations is indicated by Western blots in which the β-F_1 subunit is the dominant labeled band (Fig. 8).

Characteristics of the ATPase activities assayed in mitochondrial and peroxisomal preparations under similar conditions are all very similar. This would tend to indicate that perhaps the activity is in fact mitochondrial and not uniquely peroxisomal, if the assumption is made that there should at least be some differences in properties between such activities located in different organelles as the case with the three established yeast ATPase enzyme types. The finding that antibody against yeast mitochondrial ATPase recognizes protein bands in the peroxisomal preparations and that these bands appear to be of similar molecular mass to certain subunits of the true mitochondrial enzyme also supports the contention that perhaps there is not a unique peroxisomal ATPase. Confirmation that the ATPase activity found associated with peroxisomes is mitochondrial in origin was provided by the mitochondrial labeling experiment in which [^{35}S]methionine-labeled F_1 subunits are identified within purified peroxisomal fractions (Fig. 7). The lack of radioactivity in the alcohol oxidase band suggested that inadvertent labeling of the methanol-grown cells did not occur. Furthermore, the ratio of labeling (cpm) to the amount of pH 9.0 ATPase activity in the mitochondrial fraction is in close agreement with the same ratio in the peroxisomal material isolated from the 50/60% sucrose interface. If a unique peroxisomal enzyme were present, this ratio should have been much lower in the peroxisomal fraction. This result suggests that there is probably no mitochondrial-like ATPase associated with peroxisomes that is not mitochondrially derived.

The recently described yeast or fungal vacuolar-type ATPase was not found in our preparations. This enzyme was reported to have a pH optimum in the region of 7.0 to 7.5 [32,33], and is insensitive to both azide and vanadate. Vacuolar ATPase activity measured at pH 7.5 in the presence of azide and vanadate is also as sensitive to inhibition by DCCD as is the mitochondrial-type enzyme [30,31]. This type of activity was not found associated with peroxisomes since there was very little detectable activity at any pH when measured in the presence of both azide and vanadate (Fig. 2). Furthermore, the sensitivity profile of the peroxisomal activity to DCCD, when measured under conditions that would optimize vacuolar activity, is intermediate between that of the plasma membrane and mitochondrial enzymes (data not shown), and is not characteristic of the vacuolar enzyme as reported by others.

The presence of plasma membranes in peroxisomal preparations was indicated by a second peak of peroxisomally-associated ATPase activity at pH 6.5 that was

sensitive to vanadate. This was a surprising result in that the equilibrium density of plasma membranes from *C. tropicalis* is 1.15 g/cm^3 as reported by Blasco et al. [20] and Bowman et al. [37], and peroxisomes equilibrate to densities between 1.20–1.26 g/cm^3 [39,45,46]. Plasma membrane fragments from *S. cerevisiae*, however, differ from other species in that they apparently equilibrate below mitochondria at 1.21–1.25 g/cm^3 in sucrose gradients [16,17]. The bulk of the vanadate-sensitive activity found in our preparations of *C. boidinii* co-migrated with mitochondrial membrane markers and decreased steadily as peroxisomal membranes were purified. Vanadate-sensitive activity, in fact, has been entirely undetectable in some purifications. The small amount of activity that survived the peroxisomal membrane flotation step was virtually indistinguishable from that of the purified plasma membrane enzyme in its sensitivity to azide, venturicidin and DCCD, and similar sensitivities were found with respect to vanadate and CMP. Substrate specificities are difficult to compare in that substitutions for either Mg^{2+} or ATP decreased the pH 6.0 activity in peroxisomal membranes below detectable limits. Attempts to purify vanadate-sensitive activity from peroxisomes, however, were largely unsuccessful in that the detergents and media used were apparently insufficient either to solubilize the enzyme or to support its ATPase activity (data not shown). Mitochondrial activity, however, was the dominant H^+-ATPase in peroxisomal preparations as indicated by 3-fold greater azide-sensitive pH 9.0 activity in the 30M fraction compared to the activity at pH 6.5 with and without vanadate (Fig. 1). Competition between two contaminating species, with low specific activities, may account for small discrepancies in pH maxima and inhibitor sensitivity profiles between the peroxisomal activity and those of the purified plasma membrane enzyme. Moreover, the plasma membrane ATPase activity was assayed after solubilization of the enzyme in deoxycholate, whereas the activity associated with peroxisomes was presumably in its original membrane environment. Estimated values of K_m, likewise, are best performed on purified enzymes and seemingly unique characteristics of a peroxisomal ATPase are more likely to be complex effects of the conditions under which they were assayed.

While the possible existence of a novel vanadate-sensitive peroxisomal ATPase can not be entirely ruled out, the results of these investigations strongly suggest that both the mitochondrial and plasma membrane enzymes are persistent contaminating species in otherwise purified preparations of peroxisomal membranes. A not unreasonable conclusion from these data is that the ATPase activity found associated with yeast peroxisomes is not unique to the organelle, but is likely due in large part to contamination of these preparations by other membrane fragments.

An alternative explanation is that the same enzyme, or subunits of it, is found both in peroxisomes and in mitochondria. The immunocytochemical experiments of Douma et al. [4] tend to support such an hypothesis but final confirmation will have to await more powerful biochemical and genetic analysis. Similar results have been found in purified rat liver peroxisomes where it was estimated that only 25–35% of the ATPase activity can be attributed to contamination by other organelles. The activity was proposed to be that of a novel vacuolar/lysosomal-type ATPase also possessing some properties of the mitochondrial type enzyme [6]. The absence of a distinct peroxisomal ATPase, however, does not rule out the possible existence of an otherwise energized membrane that appears to be necessary for protein translocation and other membrane functions.

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