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Peroxisomes induced in *Candida boidinii* by methanol, oleic acid and D-alanine vary in metabolic function but share common integral membrane proteins

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Summary

Peroxisomes massively proliferate in the methylotrophic yeast *Candida boidinii* when cultured on methanol as the only carbon and energy source. These organelles contain enzymes that catalyze the initial reactions of methanol utilization. The membranes contain abundant proteins of unknown function; their apparent molecular masses are 20, 31, 32 and $47 \times 10^3 M_r$ and are termed PMP20, PMPs31–32 and PMP47. Recently, we reported that peroxisomes in this yeast are also induced by oleic acid and D-alanine as carbon sources, and that these peroxisomes contain increased concentrations of the enzymes of fatty acid β -oxidation or D-amino acid oxidase, respectively. This report extends these findings and further compares the enzyme composition from peroxisomes induced by methanol, oleic acid and D-alanine. The patterns of matrix proteins represented on SDS–polyacrylamide gels from peroxisomes induced by oleic acid or D-alanine were found to be very different from those of peroxisomes

induced by methanol. In order to differentiate between membrane proteins that have specific functions in pathways of substrate utilization from those with more generalized functions, peroxisomal membranes from cultures grown on methanol, oleic acid or D-alanine were purified. Analysis of these fractions demonstrated that while PMP20 is found only in peroxisomes induced by methanol, the PMPs31–32 and PMP47 were the abundant peroxisomal membrane proteins (PMP) regardless of inducing substrate. The data strongly suggest that the function of PMP20 is related to methanol metabolism. In contrast, the functions of PMPs31–32 and PMP47 are 'substrate-nonspecific'. We speculate that they may relate to the structure, assembly or general function of the organelle.

Key words: *Candida boidinii*, peroxisomes, membrane proteins.

Introduction

Peroxisomes (microbodies) are organelles that are ubiquitously present in eukaryotic cells. They can catalyze a variety of biosynthetic and oxidative reactions that often lead to the generation of hydrogen peroxide. The specific functions of the organelle can vary widely in different tissues or in different species; this behavior is perhaps most striking in eukaryotic microorganisms. For example, microbodies of trypanosomes, filamentous fungi and yeast can specialize in glycolysis (Oppenheimer *et al.* 1984), the glyoxylate shunt (Desel *et al.* 1982) and fatty acid β -oxidation (Ueda *et al.* 1985; Veenhuis *et al.* 1987), respectively. The differences between these organelles are much more obvious than their similarities. However, some generalizations can be made that relate to peroxisomes from diverse sources. All peroxisomes studied to date have a relatively thin membrane, are fragile *in vitro*, and contain at least residual fatty acid β -oxidation activity.

Most but not all peroxisomes contain catalase (Kionka and Kunau, 1985; Oppenheimer *et al.* 1984; Tolbert, 1981), they are usually but not always spherical (Gorgas, 1982) and most peroxisomal matrix proteins contain the sorting tripeptide SKL at their carboxyl terminus (Gould *et al.* 1988), although there are exceptions to this generalization as well (Small *et al.* 1988).

On the basis of research in other organellar systems, it is reasonable to expect that common elements exist that participate in the assembly of all, or at least a large subset of, peroxisomal proteins. While peroxisomal membrane proteins (PMPs) have been identified in several systems (Aman and Wang, 1987; Goodman *et al.* 1986; Lazarow and Fujiki, 1985), it is not clear which have restricted metabolic functions and which have a more general role in peroxisomal structure and/or assembly. The mammalian PMP70, the only peroxisomal integral membrane protein whose primary sequence has been reported has sequence similarity to the P-glycoprotein (Kamijo *et al.* 1990); this

provocative finding suggests that it provides channel or transport activity, although its precise function is not known. One way of discriminating between metabolically specialized and generalized peroxisomal membrane proteins is to compare the protein composition of peroxisomal membranes from organelles performing different functions.

We have been studying peroxisomes in *Candida boidinii*, an asporogenous yeast that induces abundant peroxisomes when provided with methanol as the only carbon source (Sahm *et al.* 1975). These peroxisomes are composed almost exclusively of two proteins, alcohol oxidase and dihydroxyacetone synthase, that catalyze the initial steps of methanol assimilation; catalase is also present as a minor constituent by mass (Goodman, 1985). Membranes stripped of most peripheral elements with sodium carbonate (Fujiki *et al.* 1982) are enriched with four abundant proteins, termed PMP20, PMPs31–32 and PMP47 (Goodman *et al.* 1986). PMP20 is found in the peroxisomal matrix as well as in the membrane (Veenhuis and Goodman, 1990), and is the most easily removed by sodium carbonate (Goodman *et al.* 1986); it probably exists in two isoforms (Garrard and Goodman, 1989). PMPs31–32 are two proteins of 31 and $32 \times 10^3 M_r$, that have a high degree of sequence similarity between them (R. Lark, C. Slaughter, C. Moomaw, J. Hsu and J.M. Goodman, unpublished data). PMP47 is most resistant to extraction from the membrane by sodium carbonate (Goodman *et al.* 1986); the protein sequence deduced from the gene includes at least two candidates for membrane-spanning domains (M. McCammon, C. Dowds, C. Slaughter, C. Moomaw, K. Orth and J.M. Goodman, unpublished data).

Among the metabolic substrates that can induce peroxisomal proliferation in various species of yeast are D-alanine (Zwart *et al.* 1983) and oleic acid (Veenhuis *et al.* 1987). Recently we have reported that *C. boidinii* can utilize these substrates as sole carbon source, and growth on these compounds is associated with the development of peroxisomes (Sulter *et al.* 1990). We now provide a detailed comparison of the protein composition of these organelles. We report that while the matrix proteins differ greatly between peroxisomes from cells cultured on oleic acid or D-alanine compared to methanol, the abundant membrane proteins PMPs31–32 and PMP47 are consistently found in the membrane. Rather than participate in a particular metabolic pathway of substrate utilization, these 'substrate-nonspecific' proteins may thus play a more general role in peroxisomal structure or function.

Materials and methods

Growth of *Candida boidinii*

The organism (ATCC strain no. 32195) was cultured in minimal medium supplemented with yeast extract (van Dijken *et al.* 1976); sodium sulfate was substituted for ammonium sulfate when cells were grown in D-alanine so that D-alanine was the only carbon and nitrogen source. In order to obtain vigorous induction of peroxisomes, cells were precultured in medium containing 2.5% glucose at 30°C for 24 h; these log-phase cells (A_{600} between 2 and 8) were then diluted 1000-fold into media with the appropriate carbon and/or nitrogen source and incubated for a further 24 h before harvesting. Concentrations of inducing substrates in the medium were 0.5% methanol (v/v), 0.1% oleic acid (w/w), or 0.5% D-alanine (Sigma). The A_{600} at harvesting was typically 0.5 to 2.

Whole cell lysates

Cell pellets (typically 50 A_{600} units) were resuspended in 1 ml of

5% trichloroacetic acid and kept on ice for at least 15 min to ensure denaturation of all proteins. Suspensions were centrifuged for 15 min in a cold microfuge, pellets were washed twice with 1 ml cold acetone, and then resuspended in 250 μ l Laemmli sample buffer (Laemmli, 1970) without Bromophenyl Blue, neutralized with a small volume of NaOH, and then 0.3 g of acid-washed glass beads were added. Samples were lysed by hand vortexing for 20–30 min and the lysates were removed from the beads. They were boiled for 3 min and then centrifuged for 3 min in a microfuge; the pellets were discarded. Protein concentrations of all lysates were determined (Schaffner and Weissmann, 1973); they were approximately 5 mg ml⁻¹.

Organelle fractionations

Peroxisomes, typically from 1 liter cultures at A_{600} 0.5–2.0, were isolated on sucrose gradients essentially as described (Goodman, 1985), but modified slightly for each inducing substrate to optimize separation of mitochondria and peroxisomes. Each gradient contained a 4 ml top layer, four intermediate layers of 6.5 ml each, and a 3 ml bottom cushion. The sucrose concentrations (all %, w/w) were 30, 33, 37, 40, 45 and 50 (glucose and D-alanine as substrates), 30, 33, 39, 42, 45 and 50 (oleic acid as substrate), or 30, 35, 40, 45, 50 and 60 (methanol as substrate). For cells grown in oleic acid, cells were washed twice in distilled water before preparation of spheroplasts in order to clear the suspension of oleic acid droplets.

Peroxisomal subfractionation

Peroxisomes isolated from a sucrose gradient were diluted 4-fold by the addition of 3 volumes cold TI 8 buffer (10 mM Tris-HCl, pH 8, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidinium-HCl, and 5 μ g ml⁻¹ of both leupeptin and aprotinin (all chemicals were from Sigma)). The suspension was centrifuged for 1 h (1.5 h for oleate peroxisomes) at 50 000 revs min⁻¹ in a Beckman Ti 70 rotor. The resulting pellet was resuspended in a minimal volume (typically 200 μ l) in cold TI 8 buffer. Six volumes of sodium carbonate were added such that the final concentration of carbonate was 0.1 M. Extraction with carbonate was allowed to proceed on ice for 1 h with occasional agitation. Membranes were then recovered by centrifugation in a Ti 70 rotor at 50 000 g for 1 h (2 h for oleate peroxisomes).

When further fractionation of the carbonate pellets was indicated, the pellets were resuspended in 300 μ l TI 8 buffer and applied to a 3.8 ml continuous 10% to 30% (w/v) sucrose gradient containing all the reagents in TI 8 buffer at identical concentrations. The gradients were centrifuged in a Beckman SW60 rotor at 2°C for 18 h at 50 000 revs min⁻¹. They were then fractionated into 300 μ l fractions, and the pellets were also resuspended in 300 μ l of TI 8 buffer. For analysis of these gradient fractions, trichloroacetic acid to 10% final concentration was added. Protein precipitates were washed twice in cold acetone and dissolved in 2× Laemmli sample buffer as described above.

Protein gels, blots, and enzyme assays

Gels (9%) (Laemmli, 1970) with a separating gel at pH 9.2 were employed. Immunoblots were performed by standard methods (Towbin *et al.* 1979); dilutions of antibodies were 1:400 for anti-alcohol oxidase antiserum (Goodman *et al.* 1984), 1:200 for monoclonal antisera IIB5 (anti-dihydroxyacetone synthase) and IIC1 (anti-PMP20), and 1:100 for IVA7 (anti-PMP47) (Goodman *et al.* 1986), unless otherwise noted. Published methods were used for the measurement of alcohol oxidase (Tani *et al.* 1985), D-amino acid oxidase (Rosso *et al.* 1969), except that the concentration of D-alanine in the reaction was 250 mM, catalase (Goodman, 1985), except the molar absorbance index of 43.6 was used for H₂O₂ (Worthington Enzyme Catalog), cytochrome c oxidase (Tolbert, 1974) and acyl-CoA oxidase (Dommes *et al.* 1981), using decanoyl-CoA as substrate.

Electron microscopy

For the detection of PMP47 by immunocytochemical means, intact cells were fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 90 min at 0°C, dehydrated in a

graded ethanol series and embedded in Lowicryl K4 M (Zagers *et al.* 1986). Polymerization was at -35°C by u.v. light. Immunogold labeling (Slot and Geuze, 1984) was performed on ultrathin sections using IVA7 monoclonal anti-PMP47 antibody and protein A/gold. For studies of cell morphology, whole cells were fixed in 1.5% (w/v) KMnO_4 for 20 min at room temperature. Isolated organelles from sucrose gradients were exposed overnight to 6% glutaraldehyde at 4°C , then diluted with 2 volumes of 6% glutaraldehyde, pelleted at $14\,000\text{ g}$ for 20 min, and stored for further processing in 0.1 M sodium cacodylate, pH 7.2, containing 6% glutaraldehyde. Postfixation was performed in a solution of 1% (w/v) OsO_4 plus 2.5% (w/v) $\text{K}_2\text{Cr}_2\text{O}_7$ in the same cacodylate buffer for 60 min at room temperature. After fixation the samples were poststained in 1% (w/v) uranyl acetate in distilled water for 8–16 h, dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

Results

Micrographs of *C. boidinii* growing in media containing various carbon sources are shown for reference in Fig. 1. Peroxisomes are small and difficult to find in cells grown on glucose (Fig. 1A, arrow). However, such organelles could be definitively identified by staining for catalase with diaminobenzidine as well as by immunodecoration with antibodies against PMP47 (Veenhuis and Goodman, 1990), a peroxisomal integral membrane protein. When cells were transferred to medium containing methanol as the sole carbon source, peroxisomes proliferated in a cluster until they encompassed 25–30% of cytoplasmic volume (Fig. 1B; and Sahm *et al.* 1975); the cluster is derived from one of a few small peroxisomes present before the addition of methanol (Veenhuis and Goodman, 1990). We have recently reported that peroxisomes are also induced in *C. boidinii* by oleic acid and D-alanine (Sulter *et*

al. 1990). Peroxisomes in cells cultured in oleic acid (Fig. 1C) were smaller and more numerous than those induced by methanol. The cells also contained abundant mitochondria, perhaps for utilization of acetyl-CoA, the product of fatty acid β -oxidation. The extent of peroxisomal proliferation in D-alanine (Fig. 1D) is less than in methanol or oleic acid.

Antibodies have been generated against the two abundant matrix proteins of the methanol-induced organelle, alcohol oxidase (Goodman *et al.* 1984) and dihydroxyacetone synthase (Goodman, 1985), as well as against two membrane proteins, PMP20 and PMP47 (Goodman *et al.* 1986). In order to gain insight into the function of the PMPs, their relative concentrations were compared by immunoblotting total cell lysates from cells grown on the carbon sources discussed above. Fig. 2 illustrates total cell lysates subjected to SDS-gel electrophoresis and Coomassie staining (A) or to immunoblotting (B). Extracts and organellar fractions (see below) from D-alanine-grown cells did not yield well-resolved protein bands on SDS-containing gels. There was very little cross-reactivity of any of the peroxisomal antibodies in the lysate derived from glucose-grown cells. In the presence of a higher concentration of anti-PMP47 antibody during the immunoblotting, a trace of this protein could be seen in the glucose lysate; in contrast, no alcohol oxidase (the faint band represents spillover in this lane and was not seen in several other experiments), dihydroxyacetone synthase or PMP20 could be visualized in this sample by immunoblotting at any concentration of antibody (Veenhuis and Goodman, 1990). As expected, all four of these peroxisomal proteins were clearly detected in lysates from methanol-grown cells. The matrix proteins dihydroxyacetone synthase and alcohol oxidase represent the two major bands at 79 and $73 \times 10^3 M_r$ visible on the Coomassie-stained gel

Fig. 1. Proliferation of peroxisomes in *C. boidinii* grown on different substrates. Electron micrographs of cells that were cultured in media containing (A) glucose, (B) methanol, or (C) oleic acid as sole carbon source, or (D) D-alanine as sole carbon and nitrogen source. Arrows point to these organelles. Note the small peroxisome in cells cultured in glucose-containing medium (A). n, nucleus. Bar, $1\text{ }\mu\text{m}$.

Carbon Source:	Glucose	Methanol			Oleate			D-Alanine		
Protein, μg :	100	100	30	10	100	30	10	100	30	10

Fig. 2. Expression of peroxisomal proteins in whole cell lysates. The indicated mass of whole cell lysates cultured as described were electrophoresed through SDS-polyacrylamide gels. Gels were (A) stained with Coomassie Blue, or (B) immunoblotted with antibodies to the indicated proteins as described in Materials and methods. DHAS, dihydroxyacetone synthase.

(Fig. 2A). In contrast to methanol-grown cells, there was no dihydroxyacetone synthase and very little alcohol oxidase present in lysates from cells cultured in oleic acid

or D-alanine. This result was not surprising, since these enzymes are required only for methanol metabolism. The detectable concentrations of alcohol oxidase in the oleic

Fig. 3. PMP47 is always found associated with the peroxisomal membrane. Immuno-electron microscopy with anti-PMP47 monoclonal antibody and detection by colloidal gold were performed. Cells were cultured in media containing the following substrates: (A) glucose; (B) methanol; (C) oleic acid; or (D) D-alanine. m, mitochondrion; n, nucleus; p, peroxisome; v, vacuole. Bar, 0.5 μ m.

acid and D-alanine cultures were probably the result of derepression of the gene in the absence of glucose (Harder *et al.* 1987). The expression of PMP20 was similar to that of dihydroxyacetone synthase: no expression was ever detected in the absence of methanol. This result suggests that the function of PMP20 is related to methanol utilization; it is a 'substrate-specific' PMP.

In contrast to PMP20, PMP47 was induced by all three of the peroxisomal proliferators. The concentration of PMP47 was even greater in cells cultured in oleic acid than in methanol (a difference of about 30-fold, determined by quantitative Western analysis using a range of sample dilutions). This may reflect in part a greater peroxisomal membrane surface area in oleic acid-grown cells; careful morphometric analysis may be required to clarify this issue. The concentration of PMP47 in cells grown on D-alanine was similar to that in cells grown on methanol. Since PMP47 was induced by these disparate metabolic substrates, we term this protein to be a 'substrate-nonspecific' PMP, in contrast to PMP20. Previous biochemical experiments have shown that PMP47 is tightly associated with the membranes of methanol-induced peroxisomes (Goodman *et al.* 1986). To confirm that PMP47 is localized in peroxisomes regardless of inducing substrate, sections of cells grown in media containing glucose, methanol, oleic acid and D-alanine were subjected to immuno-electron microscopy utilizing MAb IVA7, an anti-PMP47 monoclonal antibody (Goodman *et al.* 1986). As can be seen in Fig. 3, peroxisomes specifically reacted with this antibody in all cases.

The composition of proteins in the peroxisomal matrix and membrane of *C. boidinii* grown on methanol-containing medium has been well documented (Goodman, 1985; Goodman *et al.* 1986; Goodman *et al.* 1984). In order to compare this pattern with peroxisomes caused to proliferate by oleic acid and D-alanine, the organelles were resolved on sucrose gradients by essentially the same

method that was described for methanol-induced organelles (see Materials and methods). The densities of the sucrose layers were adjusted slightly for each carbon source in order to maximize the resolution between mitochondria and peroxisomes. Analysis from such fractionation on sucrose gradients is presented in Fig. 4. Fig. 4A shows the distribution of peroxisomal enzymes and of a mitochondrial marker, cytochrome *c* oxidase; Fig. 4B illustrates protein-stained SDS-gels of the corresponding fractions; and immunoblots for PMP47 in the fractions are shown in Fig. 4C to verify further the location of peroxisomes on these gradients. Electron micrographs of the corresponding peak peroxisomal fractions from these gradients are shown in Fig. 5.

Fractions G1 through G8 were derived from glucose-grown cells. Protein staining indicated one predominant population of organelles from the gradient, corresponding to the distribution of cytochrome oxidase, the mitochondrial marker; peroxisomal protein was poorly visualized in this gradient because of the low abundance of this class of organelles in cells grown in glucose-containing medium. Nevertheless, analysis of these fractions for peroxisomal enzymes revealed a discrete peak of catalase and acyl-CoA oxidase in fraction G6 (the interface between 40% and 45% sucrose) that was well resolved from the mitochondrial peak in fraction G2. There was no measurable activity of alcohol oxidase in this gradient, consistent with an absence of immunoblotting reactivity shown in Fig. 2. When these fractions were tested for PMP47 protein, a weak signal was detected (Fig. 4C, fraction G6), confirming that peroxisomes indeed migrated to this fraction. Fig. 5A shows an electron micrograph of fraction G6; some peroxisomes were visible (arrow), but mitochondria were more common, as expected. This fraction (as well as the corresponding fraction from cells grown on D-alanine, Fig. 5D), also show an abundance of structures that we interpret to be fragments of cell wall. This material

Fig. 4. Fractionation of organelles on discontinuous sucrose gradients. Gradient fractions containing organelles derived from cells grown on media containing glucose (G1-G8), methanol (M1-M8), oleic acid (O1-O8), or D-alanine (A1-A8) were analyzed. (A) Activities of the indicated enzymes were measured and expressed in terms of the total recovered activities in the gradients. The values of 100% for all of the graphs in (A) are given in parentheses in Table 1. (B) Protein profiles of gradient fractions were analysed on SDS-polyacrylamide gels. For the samples from methanol- and oleic acid-grown cells, 5% of each fraction was subjected to

electrophoresis and stained with Coomassie Blue. For the glucose and D-alanine samples, 1% of each fraction was used and stained with silver; loading more than 1% of samples G6 or A6 (approximately 1 μ g of protein) resulted in blurring and altered migration of all the protein bands (see Results). Migrations of molecular weight markers are indicated to the left of each gel. (C) Five per cent of each fraction was applied to gels and immunoblotted with anti-PMP47 monoclonal antibody. A 1:50 dilution of the antibody was used for G1-G8 to visualize the immunoreactive material; a 1:100 dilution was used for the other samples.

Fig. 5. Electron micrographs of peroxisomal fractions. Electron micrographs were prepared from the following fractions of the gradients illustrated in Fig. 4: A, G6; B, M7; C, O7; or D, A6. Arrows in A and D indicate peroxisomes. Bar, 0.5 μ m.

interfered with the proper migration of proteins on SDS gels unless very low amounts of protein were loaded (notice the distortion of PMP47 on the immunoblot in lane G6 of Fig. 4C). Because the peroxisomes were so few, no attempt was made to purify them further.

Fig. 4, fractions M1 through M8, show the results of organellar fractionation of methanol-grown cells. As reported earlier (Goodman *et al.* 1984), peroxisomes sedimented at the 50%–60% sucrose interface, well separated from mitochondria. The abundant peroxisomal proteins in the SDS-gels (fraction M7) at 73 and $78 \times 10^3 M_r$ represent monomers of the matrix constituents alcohol oxidase and dihydroxyacetone synthase, respectively, (Goodman, 1985). Acyl-CoA oxidase was detected in these peroxisomes, although the activity was only about 2% of that seen in peroxisomes obtained from cells grown in oleic acid (see Table 1). PMP47 fractionated with the peroxisomal peak, as expected. Fig. 5B shows that this fraction is highly purified.

The results of a fractionation of organelles from cells grown on oleic acid is also shown in Fig. 4 (fractions O1 through O8). Peroxisomes sedimented to the 45% to 50% sucrose interface (an intermediate density between glucose peroxisomes and methanol peroxisomes) as judged by marker enzymes, and they had a complement of abundant proteins that was distinct from those in methanol peroxisomes (compare the protein staining pattern in lanes M7 and O7). This was not an unexpected finding, considering the low expression of alcohol oxidase and the absence of dihydroxyacetone synthase in lysates from cells cultured on oleic acid, shown in Fig. 2. Also as expected, acyl-CoA oxidase, a marker of the peroxisomal β -oxidation pathway that is required for growth on oleic acid, was in higher

concentration (a 370-fold increase in specific activity) in the oleic acid-induced peroxisomes compared to the methanol-induced peroxisomes (see Table 1). We did not attempt to identify specific enzymes of β -oxidation with specific bands on SDS-gels. PMP47, as visualized on the corresponding immunoblot, was seen to comigrate with the peroxisomal enzyme markers in the gradient. These oleic acid-induced peroxisomes were highly purified (Fig. 5C), and they appeared remarkably intact.

The behavior of peroxisomes from cells grown on D-alanine is shown in the last set of panels in Fig. 4. The density of the D-alanine-induced peroxisomes was very similar to the peroxisomes of glucose-grown cells; both populations migrated to the 40%–45% sucrose interface in the gradients. The protein composition of these peroxisomes was noticeably different from those of both methanol- and oleate-grown cells (compare M7, O7 and A6 in Fig. 4B). D-Alanine is metabolized to pyruvate by D-amino acid oxidase, an enzyme that is induced by D-alanine (Sulter *et al.* 1990); this activity comigrated with peroxisomes (Fig. 4A). The fraction at the 40% to 45% interface of these gradients also contains material that interferes with clear banding patterns on gels, thus interfering with a further analysis of proteins from D-alanine peroxisomes. This is also apparent by the blurred morphology of the blotted PMP47 band in fraction A6. Fig. 5D demonstrates the existence of peroxisomes in this fraction (arrows), as well as the structures resembling cell wall fragments that was also seen in fraction G6. (These copurifying structures are not present in the more-dense peroxisomes induced by methanol or oleic acid.)

A comparison of peroxisomal enzyme concentrations from the peak peroxisomal fractions of all four gradients is

shown in Table 1. Both specific activities in the peroxisomal fraction and total activities in the gradient (parentheses) are represented. Although the peroxisomal fraction from the glucose cultures was heavily contaminated with mitochondria (Fig. 5A), certain conclusions can be drawn from these data. As expected, alcohol oxidase, acyl-CoA oxidase, and D-amino acid oxidase were found in greatest concentration in the peroxisomes from methanol-, oleic acid- and D-alanine-grown cells, respectively. Catalase was at its highest concentration in peroxisomes from cells grown on oleic acid, but was induced by methanol and D-alanine, as well. This result was also expected, since oxidases (alcohol oxidase, acyl-CoA oxidase, and D-amino acid oxidase) generate hydrogen peroxide. Surprisingly, acyl-CoA oxidase was strongly induced by D-alanine as well as by oleic acid. Cells growing on D-alanine as the sole carbon and nitrogen source have no known requirement for β -oxidation; the mechanism and reason for this effect is not clear.

In order to examine the composition of the peroxisomal membranes, purified organelles from methanol-, oleic acid- and D-alanine-grown cultures were first lysed by addition of Tris-HCl buffer, pH 8.0. This has been shown to release 80–95% of alcohol oxidase and dihydroxyacetone synthase from methanol peroxisomes (Goodman *et al.* 1986); it also released a subset of proteins in the fractions containing peroxisomes from oleic acid- and D-alanine-grown cells (data not shown). The resulting membrane pellets were then incubated in 0.1 M sodium carbonate, pH 11.4, a treatment that strips membranes of peripheral proteins (Fujiki *et al.* 1982). The results for membranes derived from methanol and oleate organelles are shown in Fig. 6 (distinct patterns on SDS-gels from D-alanine-derived peroxisomes could not be obtained). As seen before for methanol peroxisomes (Goodman *et al.* 1986), PMPs 47, 31–32 and 20, as well as residual alcohol oxidase and dihydroxyacetone synthase, were the most abundant constituents of the membranes after carbonate treatment. Most of PMP20, however, was removed by this treatment, while nearly all of PMP47 (see blot in Fig. 6B) and PMPs31–32 remained. The corresponding samples from oleic acid-grown cells were more complex. However, the proteins that were most resistant to carbonate extraction were PMP47 (confirmed by immunoblotting, Fig. 6B) and three proteins of apparent molecular mass of $31\text{--}34 \times 10^3 M_r$.

Peroxisomal membranes from cultures grown on methanol, oleic acid and D-alanine, after treatment with sodium carbonate, were further purified on sucrose density gradients to separate them from protein aggregates or any contaminants (such as the cell wall fragments in the D-alanine peroxisome fraction; see Fig. 5D). The results of this experiment are shown in Fig. 7. Peroxisomal membranes migrated as a broad peak in these gradients. As expected and shown in Fig. 7A, this peak consisted of

Fig. 6. Carbonate extraction of proteins from the membranes of peroxisomes of methanol- and oleic acid-grown cells. Membranes from purified peroxisomes that had been lysed by treatment at pH 8.0 were subjected to extraction with sodium carbonate, pH 11.4, and then centrifuged to generate supernatants and pellets. Samples were subjected to SDS-gel electrophoresis and either (A) stained with Coomassie Blue or (B) immunoblotted with anti-PMP47 antibody. Lanes 1–3, samples from a methanol culture; lanes 4–6, samples from an oleic acid culture. Lanes 1 and 4, total samples after treatment with carbonate; lanes 2 and 5, supernatants; lanes 3 and 6, pellets. Molecular mass standards are indicated to the right of the gel. DHAS, dihydroxyacetone synthase; AO, alcohol oxidase.

PMPs 47, 31–32 and 20 when membranes from methanol peroxisomes were applied. Occasionally a small fraction of PMP20 migrated elsewhere in the gradient; since it is a protein found in both the matrix and peripherally on the membrane, this was attributed to aggregation or release from the membrane. The pattern for oleate membranes is shown in Fig. 7B. Proteins assumed to be PMP47 and PMPs31–32 (identical migrations on SDS-gels) were the principal constituents of the fractions in the gradient. The identity of PMP47 was confirmed by immunoblots (not

Table 1. Specific activities of induced enzymes in peroxisomal fractions from cells grown on different media

	Alcohol oxidase	Acyl-CoA oxidase	D-aa oxidase	Catalase
Glucose	<0.0005 (1.5×10^{-6})	0.07 ± 0.0012 (0.021)	<0.0002 (n.d.)	4.08 ± 2.19 (2.07)
Methanol	1.36 ± 0.156 (1.4×10^{-3})	0.07 ± 0.0176 (0.029)	0.00028* (n.d.)	235 ± 0.66 (88.11)
Oleic acid	0.01 ± 0.0039 (6.1×10^{-6})	6.53 ± 0.716 (1.28)	0.0221 ± 0.0082 (n.d.)	1201 ± 368 (192)
D-Alanine	0.0029 ± 0.0003 (1.3×10^{-6})	1.04 ± 0.166 (0.309)	0.924 ± 0.094 (0.263)	475 ± 37 (164)

The peak peroxisomal fraction from sucrose gradients (see Fig. 4) were assayed for the enzymes listed and expressed as ($\mu\text{mol min}^{-1}$) mg^{-1} protein. The values shown are mean $\pm$ range for two independent experiments, except for D-amino acid oxidase in the methanol preparation (*) where only a single experiment was used. The values of total activities/total protein recovered in the gradients, in units per mg, are shown in parentheses. D-aa, D-amino acid; n.d., not determined.

Fig. 7. PMP47 and PMPs 31–32 are the common protein constituents of carbonate-extracted membranes. The pellets following carbonate extraction of peroxisomal membranes were resuspended and subjected to isopycnic sucrose gradients, as described in Materials and methods. Fractions were concentrated by precipitation with trichloroacetic acid, resuspended in Laemmli sample buffer, and electrophoresed; the gels were stained with silver. The first lane of each gel represents the pellets of these gradients; subsequent lanes are the gradient fractions, from bottom to top (left to right). Peroxisomal membranes are represented from cells grown in the following substrates: A, methanol; B, oleic acid; and C, D-alanine. Gradients were 10% to 30% (w/w) sucrose for A and B; 15% to 35% sucrose for C. While the position of the peak in the gradients varied somewhat between experiments, note that PMPs31–32 and PMP47 always comigrated and peaked in the same fractions.

shown). However, there was usually a denser fraction in the gradient (lane 2) that contained these and other proteins, including the $34 \times 10^3 M_r$ protein seen in Fig. 6 (the most slowly migrating band of the $31\text{--}34 \times 10^3 M_r$ triplet). This fraction may represent a subpopulation of peroxisomal membrane containing some aggregated matrix protein, although its distinct migration in this gradient was surprising and unique to oleic acid-induced peroxisomes.

The corresponding gradients from a preparation derived from D-alanine-grown cells is shown in Fig. 7C. The composition of fraction 1 (the pellet of this gradient) could not be determined on SDS-gels but most likely reflects the contaminating cell wall fragments seen with the peroxisomes. However, proteins that migrated identically to PMPs31–32 and PMP47 (and confirmed to be PMP47 with antibody; data not shown) were clearly the major proteins in the broad membrane peak in the gradient. Antibodies will be required to confirm the identity of the PMPs31–32 in panels B and C, although they migrate identically to their methanol counterparts on SDS-gels (Fig. 6 and data not shown).

Discussion

We have compared the protein composition of peroxisomes induced to proliferate by three disparate carbon sources: methanol, oleic acid and D-alanine. Unlike proliferators of mammalian peroxisomes such as the hypolipidemic drugs and phthalate-ester plasticizers, all of which induce a similar pattern of peroxisomal enzymes (Reddy *et al.* 1987), the substrates utilized in this study led to the development of different constellations of peroxisomal matrix proteins. This was the anticipated result, since the enzyme systems for utilization of these substrates are distinct. Thus, alcohol oxidase and dihydroxyacetone synthase are the initial enzymes of methanol assimilation (Goodman, 1985), the components of β -oxidation of fatty acids are required for oleate metabolism, and D-amino acid oxidase, a known peroxisomal enzyme in yeast (Zwart *et al.* 1983), is required for conversion of D-alanine to pyruvate. However, while the matrix protein composition was indeed different in peroxisomes from cells grown in different media, we have shown that the integral membrane proteins of all these peroxisomes were PMP47 and the PMPs31–32. Since methanol, oleic acid and D-alanine indeed caused the induction of different sets of matrix proteins, we suggest that the functions of PMPs31–32 and PMP47 are not directly involved with one pathway of substrate utilization.

Our experiments indicated that PMP20, the most abundant membrane-associated protein in peroxisomes from cells grown on methanol (Goodman *et al.* 1986), is induced only by methanol; its regulation, and that of dihydroxyacetone synthase, are more tightly controlled than that of alcohol oxidase (Fig. 2), since neither of these proteins could be detected in organelles from oleate- or D-alanine-grown cells. These data suggest that the role of PMP20 is involved with methanol metabolism, although its exact function in this utilization pathway is not known. There are two genes that encode slightly different isoforms of PMP20; their transcription is tightly regulated by methanol (Garrard and Goodman, 1989). As shown in Fig. 6 and in previous work (Goodman *et al.* 1986), the protein is preferentially bound to the membrane following peroxisomal lysis at pH 8.0 but is in large part extracted

by sodium carbonate. Immuno-electron microscopy indicates that a significant fraction of this protein is in the matrix (Veenhuis and Goodman, 1990). These data are consistent with the absence of a good candidate for a transmembrane spanning domain in the predicted PMP20 protein sequence (Garrard and Goodman, 1989), which is further evidence that the protein is only peripherally associated with the membrane.

PMP47 has recently been cloned and sequenced (M. McCammon, C. Dowds, C. Slaughter, C. Moomaw, K. Orth and J.M. Goodman, unpublished data). As expected from its tight association with the membrane (Figs 6 and 7), it contains two candidates for membrane-spanning domains. Its role remains obscure, however, since no homology with any protein sequence in data bases could be drawn. The ability to identify a homolog to PMP47 in *Saccharomyces cerevisiae* would permit a genetic approach to studying the function of this protein. Unfortunately, no homologous protein in the yeast has been found by immunoblotting with monoclonal or polyclonal antibodies against the *Candida* protein, and there is no abundant protein in peroxisomal membranes from *S. cerevisiae* of similar mass. Therefore, it is possible that PMP47 has no homologs in other yeasts.

The DNA sequences encoding PMPs31–32 are currently being determined from genomic clones. The sequences of tryptic fragments from isolated PMP31 and PMP32 indicate that these proteins are very similar but non-identical (R. Lark, J. Hsu, C. Moomaw, C. Slaughter and J. M. Goodman, unpublished results). Unlike PMP47, proteins of similar apparent molecular masses to PMPs31–32 are abundant constituents of the peroxisomal membrane from *S. cerevisiae* (M. McCammon, M. Veenhuis, S. Trapp and J. M. Goodman, unpublished data). The isolation of the genes encoding these homologs is in progress in order to be able to genetic approaches to determine their function.

What are possible roles for PMPs31–32 and PMP47? These proteins may catalyze the translocation of peroxisomal metabolites, either by serving as either transporters or pores (Lemmens *et al.* 1989; Van Veldhoven *et al.* 1987). The expression of PMP47 may be correlated to fatty acid oxidation in peroxisomes; highest concentrations of both PMP47 and acyl-CoA oxidase exist in oleic acid-grown cells. However, PMP47 is clearly induced over 100-fold in methanol-grown cells compared to glucose-grown cells (Veenhuis and Goodman, 1990); a similar induction of acyl-CoA oxidase is not caused by methanol. Considering the metabolism of methanol, fatty acids and D-alanine by peroxisomes, the common steps shared by these substrates involve oxidation by flavoenzymes and degradation of hydrogen peroxide by catalase, a heme-containing protein. It is not known how heme or flavin cofactors cross membranes. It is possible that one role of these PMPs is to catalyze this process.

It is also possible that these PMPs are playing a structural role in the organelle. They may be involved with maintaining the size and shape of the organelle, or in changing the shape during peroxisomal proliferation. Future experiments in which the expression of these proteins are altered in an heterologous system will test these possibilities.

A more provocative yet equally plausible possibility is that one or more of these PMPs mediates protein sorting or translocation. There has not been any protein or complex identified to date that performs these functions in the peroxisomal membrane; it is not clear whether all peroxisomal matrix proteins utilize a single species of 'translo-

con'; the carboxy-terminal SKL motif is not seen on all peroxisomal matrix proteins (Gould *et al.* 1989). We hope to address this hypothesis by studying the role of homologous PMPs in the genetically manipulable yeast, *S. cerevisiae*.

It is interesting to consider how PMPs31–32 and PMP47 themselves assemble in the peroxisomal membrane. There is evidence that peroxisomal membrane proteins are synthesized on soluble polyribosomes and that they assemble post-translationally (Fujiki *et al.* 1984; Suzuki *et al.* 1987). They are presumed to assemble directly from the cytoplasm into peroxisomes, as do matrix proteins. However, membrane proteins may utilize an assembly machinery that is distinct from that for the matrix proteins, since they sort into peroxisomal 'ghosts' in Zellweger fibroblasts that contain no intact peroxisomes, while matrix proteins stay in the cytoplasm or are degraded (Santos *et al.* 1988). Such ghosts also are seen in peroxisomal mutants of *Hansenula polymorpha* (Cregg *et al.* 1989). Furthermore, neither mammalian PMP70 nor yeast PMP47 end in the sorting tripeptide SKL (Kamijo *et al.* 1990; M. McCammon, C. Dowds, C. Slaughter, C. Moomaw, K. Orth and J.M. Goodman, unpublished data). The solution to this conundrum must await further analysis of membrane protein sorting.

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