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Intracellular Fate of Hydrocarbons

Possible Existence of Specific Compartments for Their Biodegradation

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Abstract

In previous work, purification procedures and zymogram analysis conducted with supernatants of crude extracts from aerobic mycelium of the YR-1 strain of *Mucor circinelloides* isolated from petroleum-contaminated soils indicated the existence of only one soluble alcohol oxidase (sAO) activity. In the present work enzymatic activity of alcohol oxidase (AO) was also detected in the mixed membrane fraction (MMF) of a high-speed centrifugation procedure after drastic ballistic cellular homogenization to break the mycelium from strain YR-1. When mycelial cells were gently broken by freezing the mycelium with liquid nitrogen, smashing in a mortar, and submitting the samples to an isopycnic sucrose gradients (10–60% sucrose), AO activity was detected in particular and discrete fractions of the gradient, showing specific density values quite different from the density of peroxisomes. The results suggest that there could be a different intracellular pattern of distribution of the microsomal fraction in aerobically grown mycelium depending on the carbon source used in the culture media, including alcohols and hydrocarbons, but not in glucose. In working with particulate fractions, we found two AO activities: a new membrane alcohol oxidase (mAO) activity and the sAO. Both activities appear to be located in the inner of the cells in specific compartments different from the peroxisomes, so mAO could be in the membrane of these compartments and sAO in the lumen of the vesicles. We also assayed other enzymatic activities involved in hydrocarbon biodegradation to establish its intracellular location and other enzymatic activities such as peroxidase to use them as intracellular markers of different organelles. In the case of monooxygenase, the first enzymatic step in the hydrocarbon biodegradation pathway, its location was in the same fractions where AOs were located, suggesting the existance of a specific organelle that contains the enzymatic activities involved in hydrocarbon biodegradation.

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Index Entries: Filamentous fungi; hydrocarbon biodegradation; petroleum contamination; microsomes; peroxisomes; alcohol oxidase.

Introduction

Most industrial chemicals are currently produced from petroleum and also hydrocarbon, representing an enormous energy resource without which our modern lifestyle would be impossible. Principally exploited as fossilized fuels, hydrocarbons must be considered a finite resource, although methane and lignin are certainly renewable. Hydrocarbons are also an important feedstock for the chemical industry. Unfortunately, hydrocarbon compounds are one of the major environmental pollutants as a result of improper disposal processes or spills of petroleum or petroleum-derived products. The most important classes of organic pollutants are mineral oil constituents and halogenated products of petrochemicals. These facts and the continuous increase in the number of toxic compounds generated by the oil industry have emphasized the importance of the development of effective processes to eliminate these waste products *(1)*.

In nature, the most rapid and complete degradation of the majority of pollutants is brought about under aerobic conditions. Therefore, the capacities of aerobic microorganisms are of particular relevance for the biodegradation of such compounds, and they are exemplarily described with reference to the degradation of aliphatic and aromatic hydrocarbons. There exist numerous types of microorganisms useful in the biodegradation processes of these hazardous materials, and many of them are capable of using hydrocarbons as the only carbon and energy source; however, when the number of carbon atoms in the hydrocarbon chain is increased to a certain amount, only several microorganisms are capable of metabolizing the hydrocarbon chains *(2)*. A huge number of bacterial and fungal genera possess the capability of degrading organic pollutants. Enzymatic key reactions of aerobic biodegradation are oxidations catalyzed by oxygenases and peroxidases. Oxygenases are oxidoreductases that use O_2 to incorporate oxygen into the substrate. Degradative microorganisms need oxygen at two different metabolic sites: at the initial attack of the substrate and at the end of the respiratory chain. The first step in aliphatic-hydrocarbon biodegradation is catalyzed by an oxygenase named cytochrome P-450 (Cyt-P450) followed by the action of an alcohol oxidase (AO) *(3)*. This enzyme is capable of using-a wide range of xenobiotic compounds as substrates, leads to the production of corresponding alcohols; Cyt-P450 could be founded in microorganisms as well as in plants and animals *(4)*. The oxidation of alcohols to the corresponding aldehyde is catalyzed by AO; aldehyde is in turn converted into the corresponding carboxylic acid *(5)*. The reactions catalyzed by Cyt-P450 and AO are special points for bioremediation chemistry. So far

most of the studies regarding the role of AO in hydrocarbon metabolism have been conducted on bacterial strains, and in several cases, AO enzymes from eukaryotic origin with physiologic roles related to hydrocarbon metabolism have been reported *(6)*. In particular, the intracellular location of these enzymatic systems in fungi is little understood, and this represents an interesting point in the knowledge of the intracellular fate of the hydrocarbons.

In the present study, we continue the studies on AO activity in *Mucor circinelloides* strain YR-1. We describe some differences in the intracellular pattern in the location of the enzymatic activities of monooxygenase and AO involved in the first and second steps in aliphatic hydrocarbon biodegradation by the presence of glucose or hydrocarbons in culture media. The results suggest that monooxygenase and AO could be located in some kind of particular microsomal vesicles different from the peroxisomes and their presence depends on the hydrocarbons used as the sole carbon source in culture media.

Materials and Methods

Chemicals

Phenylmethylsulfonyl fluoride (PMSF) and yeast AO were purchased from Sigma (St. Louis, MO), and the alcohol substrates were from J.T. Baker (Phillipsburg, NJ). All other reagents were of the highest purity commercially available. Protein was measured by the method of Lowry using bovine serum albumin as the standard *(7)*.

Organisms and Culture Conditions

The isolation of filamentous fungi able to grow on hydrocarbons was performed using petroleum-contaminated soil samples collected from our neighboring Salamanca refinery (Guanajuato, México). The isolates were named YR, and the particular strain used was named YR-1 and by mole cular procedures was identified as *M. circinelloides*; in all cases colonial and microscopic morphologies were established as criteria for the assessment of the isolated strains as filamentous fungi. As a wild-type AO-proficient (AO^+) organism, we used strain R-25 of *Hansenula polymorpha (8)*. Yeast-peptone-glucose (YPG) complete medium *(9)*, and salts minimal medium with 0.1% peptone added (sMMP) containing the specified amounts of glucose or hydrocarbons as carbon sources, were used to cultivate the fungus. Strains were maintained in agar slant tubes, and spores were obtained after grown in YPG medium as described previously *(9)*. Liquid cultures (600 mL) were propagated in 2-L Erlenmeyer flasks inoculated with spores at a final cell density of 5×10^5/mL and incubated in a reciprocating water bath shaker at 28°C for different periods of time. To obtain the aerobic mycelium, spores were inoculated in YPG medium or sMMP supplemented

with glucose (0.1%), decane (1.0%), or hexadecane (1.0%), and the cultures were incubated aerobically *(9)*.

Preparation of Cell-Free Extracts

Mycelial cells were processed and broken as described by Torres-Guzman et al. *(10)* with some modifications. Briefly, mycelial cells were washed and suspended in buffer TP8.5 (20 m*M* Tris-HCl [pH 8.5] containing 1 m*M* PMSF). A volume of about 20 mL of cells was mixed with an equal volume of glass beads (0.45–0.50 mm in diameter) and disrupted in a Braun Model MSK cell homogenizer (Braun, Melsungen, Germany) by four 30-s periods with each one under a stream of CO_2. The homogenate was centrifuged at 4360*g* for 10 min to remove cell walls and unbroken cells. The cell wall–free supernatant (crude extract) was centrifuged at 164,500*g* for 45 min; the resulting pellet, a mixed membrane fraction (MMF) (exhaustively washed), and the 164,500*g* supernatant (cytosolic fraction) were saved for enzymatic determinations. For gentle homogenization of the mycelial cells, we used essentially the same protocol; briefly, the cells were frozen by liquid nitrogen and broken in a mortar maintaining very low temperature. The broken cells were suspended in buffer TP8.5 (20 m*M* Tris-HCl [pH 8.5] containing 1 m*M* PMSF) and then centrifuged using an isopycnic sucrose gradient (20–60% [w/v]). Fractions of the gradient were collected and enzymatic activities assayed.

Enzyme Assays

AO activity was measured according to Jansen et al. *(11)*. The enzymatic assays were performed at 25°C in reaction mixtures with a 1.0-mL total volume containing 780 µL of reactive A [1.2 mL of 0.2 *M* potassium-phosphate buffer, pH 7.5; 10 µL of 1.0% *o*-dianisidine dissolved in 0.025 *M* HCl]; 5 µL of 3% peroxidase (0.01% final concentration); 150 µL of 0.2 *M* potassium-phosphate buffer; 15 µL of substrate (hexadecanol, decanol, or methanol); and 50 µL of cell-free extract (100–200 µg of protein). The reaction was started after adding substrate and the development of color measuring the absorbance at 460 nm in a Beckman DU-650 spectrophotometer. In experiments in which the pH of the reaction was varied, phosphate (50 m*M*) and Tris-HCl (50 m*M*) buffers were employed. One unit of enzyme activity was defined as the amount of enzyme that led to the production of 1 µmol of H_2O_2/min at 25°C. The specific activity of AO was expressed as units per milligram of protein. Detection of AO activity in zymograms was performed by nondenaturing polyacrylamide gel electrophoresis (PAGE) following a variation of a spectrophotometric method *(11)*. Briefly, after nondenaturing 6% (w/v) PAGE, the gel was submerged in the following solution: 4 mL of 0.2 *M* potassium-phosphate buffer, pH 7.5; 4 mL of 0.04% peroxidase; 0.4 mL of 0.01%

o-dianisidine; 0.4 mL of substrate (methanol, ethanol, hexadecanol, and so on); and 31.8 mL of H_2O. After incubating at 25°C for 60 min with gentle shaking, AO electromorphs were observed as brown bands. Oxygenase activity was assayed as described previously by Zazueta-Sandoval et al. *(12)*.

Electrophoresis

PAGE analysis of protein from different samples was carried out in slab gels using 6% (w/v) polyacrylamide with the buffer system of Laemmli *(13)*. After electrophoresis, proteins were visualized in the gels by Coomassie Blue R-250 staining (Sigma).

Immunoblotting and Immunodetection

After sodium dodecyl sulfate (SDS)-PAGE, proteins were transferred to a nitrocellulose membrane in a Mighty Small Transphor unit (Hoeffer TE22; Pharmacia Biotech, San Francisco, CA). Detection was done with polyclonal antibodies raised against purified AO from YR-1 strain and revealed with a second antibody coupled to peroxidase using 3,3′-diaminobenzidine (Sigma).

Results

Subcellular Distribution of AO

AO was detected in cell-free extracts of strain YR-1 when it was grown on different carbon sources in both the pellet and supernatant of high-speed centrifugation fractions, suggesting the possible existence of two AO activities: one of them membrane associated and the other in soluble form. The activity of this enzyme was detected only when strain YR-1 was grown in the presence of hydrocarbons or alcohols as the sole carbon source but not in glucose. Table 1 shows the soluble AO (sAO) activity, as well as a new AO activity that perhaps is an integral part of the internal membrane system of the microorganism.

AO Activity in Cell-Free Extracts from Mycelial Cells

To investigate the localization of sAO and mAO, we tried different procedures to break the cells gently. Finally, we decided to freeze the cells with liquid nitrogen and then homogenize the cells by means of a mortar maintained at low temperature. The homogenate was resuspended in a minimal volume of 50 m*M* Tris-HCl, pH 8.5, with added sucrose (10%), and the presence of AO activity was analyzed by zymograms in nondenaturing 6% PAGE using different cell fractions (164,500*g* supernatant and pellet). As can be seen in Fig. 1, the band of AO activity was in the upper part of the gel only in the pellet samples, suggesting that with this type of gentle cell homogenization the enzymatic activity remains in its

Table 1
AO Activity of YR-1 Strain Grown in Different Culture Media[a]

	AO activity[b]				
Sample	Decane	Hexadecane	Decanol	Methanol	Glucose
Pellet (MMF)	1.0	0.88	0.55	0.91	0.0
Supernatant (cytoplasm)	0.28	0.43	0.71	0.11	0.0

[a]The enzyme activity was determined in the 164,500*g* pellet and supernatant from mycelial cells grown in the indicated carbon sources. Mycelial cells were drastically broken (Braun), and the MMF and cytosolic fractions were obtained by high-speed centrifugation. AO was measured with decanol as the substrate, as described in Materials and Methods. The values are the means of three independent experiments with triplicate determinations in each case.

[b]Relative activity. Pellet in decane: 0.979 U = 1.0.

compartment and that both AO activities are not soluble in the cytoplasm of the cell, perhaps because they are particulate in a specific compartment. With the present cell homogenization procedure, those compartments are not broken and the AO does not appear in the soluble fraction, as found in previous studies *(14)*. For example, Alvarado-Caudillo et al. *(14)*, working with a high-speed supernatant fraction from cells broken by a drastic ballistic method, detected the sAO activity as a soluble enzyme activity. However, by employing a gentle procedure to obtain the different cell fractions by sucrose isopycnic gradient, the AO activity could be particulate in the aforementioned compartments, located in their lumen, in contrast to when the cell homogenization is drastic and this activity appears in the cytoplasmic fraction.

Subunit Conformation of AO

In previous work, we reported that the sAO activity was conformed by homologous subunits of 46 kDa *(15)*. It was now necessary to investigate whether the two activities detected (pellet and supernatant in Table 1) could be conformed by the single 46-kDa subunit previously reported. Therefore, we conducted immunodetection experiments after SDS-PAGE for each sample. Figure 2A shows the results of samples from mycelia grown in hexadecane as the sole carbon source. As can be seen, lane 2 of Fig. 2A, which corresponds to a supernatant of a low-speed centrifugation (6450*g*) and the heterologous immunodetection, reveals the presence of two different bands, one with a molecular mass of 46 kDa, which could be the subunit of the sAO and, interestingly, a faint second band with a molecular mass of 34 kDa. The low-speed supernatant was subjected to high-speed centrifugation (164,500*g*). Lanes 3 and 4 in Fig. 2A show the

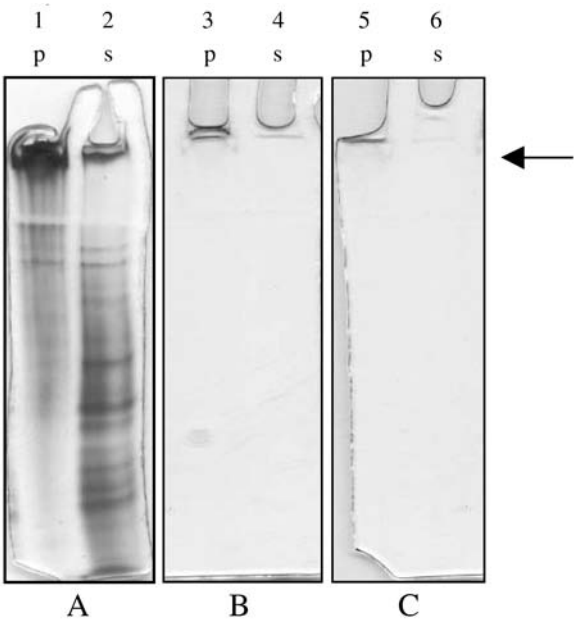

Fig. 1. Native AO detection by zymogram in different cellular fractions. Mycelia grown in different carbon sources were gently broken (liquid nitrogen and mortar), and samples of the pellet (p) and supernatant (s) from the 164,500*g* centrifugation were submitted to 6% PAGE. (A,B) Cells grown in decane as sole carbon source; (C) cells grown in methanol as sole carbon source. Lanes 1 and 2, protein pattern stained by Coomassie; lanes 3–6, zymograms of AO activity revealed with decanol as enzyme substrate. All lanes were loaded with 100 μg of protein. The arrow indicates the AO activity bands.

detection by the antibody of the ADH in the pellet and supernatant, respectively. In this case, the pellet was washed exhaustively with cell homogenization buffer before SDS-PAGE. In this sample, we detected the same two anterior bands (46 and 34 kDa). The first appears more diffuse than in the low-speed supernatant, perhaps because its concentration was lowered by the wash procedure. By contrast, the second band remains in the pellet and is more evident and easily seen than in the low-speed supernatant. Lane 4 of Fig. 2A, the sample loaded corresponding to the high-speed supernatant, shows the presence of only the 46-kDa band. These results reveal the presence of two different subunits possibly belonging to different AO enzymes. Figure 2B shows similar experiments with cell extracts from different carbon sources in the growth media. The results for mycelia grown in methanol (lanes 2 and 3) or decane (lanes 4 and 5) show a similar immunodetection pattern than in hexadecane: two bands in pellets and only one band in supernatants. These results are consistent with the idea of two different AO activities, each consisting of only one of the subunits *(14,15)*.

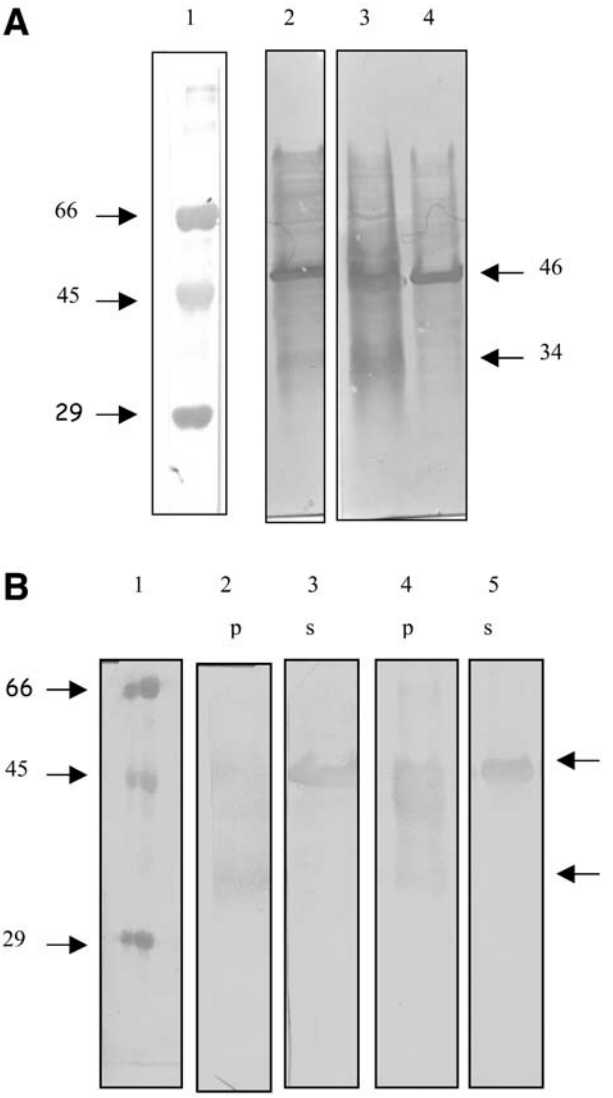

Fig. 2. Homologous immunodetection of AO subunits in different cellular fractions from mycelia grown in different carbon sources. Samples of pellet (p) and supernatant (s) from the 164,500*g* centrifugation were submitted to SDS-PAGE (8% in **[A]** and 6% in **[B]** and then transferred to a nitrocellulose membrane **(A)** Mycelia grown in hexadecane. *Lane* 1, molecular weight markers; *lane* 2, supernatant of 6450*g* centrifugation *lane* 3, Pellet of 104,500*g* centrifugation (exhaustively washed); *lane* 4, supernatant of 104,500*g* centrifugation **(B)** *Lane* 1, molecular weight markers; *lanes* 2 and 3, mycelia grown in methanol; lanes 4 and 5, mycelia grown in decane. Arrows indicate the immunodetection bands.

Intracellular Localization of AO Activities

To investigate whether AO activities could be localized in a particular microsomal body in YR-1 cells, we ran several sucrose isopycnic gradients (10–60% [w/v]) using samples of mycelia of strain YR-1 grown in different

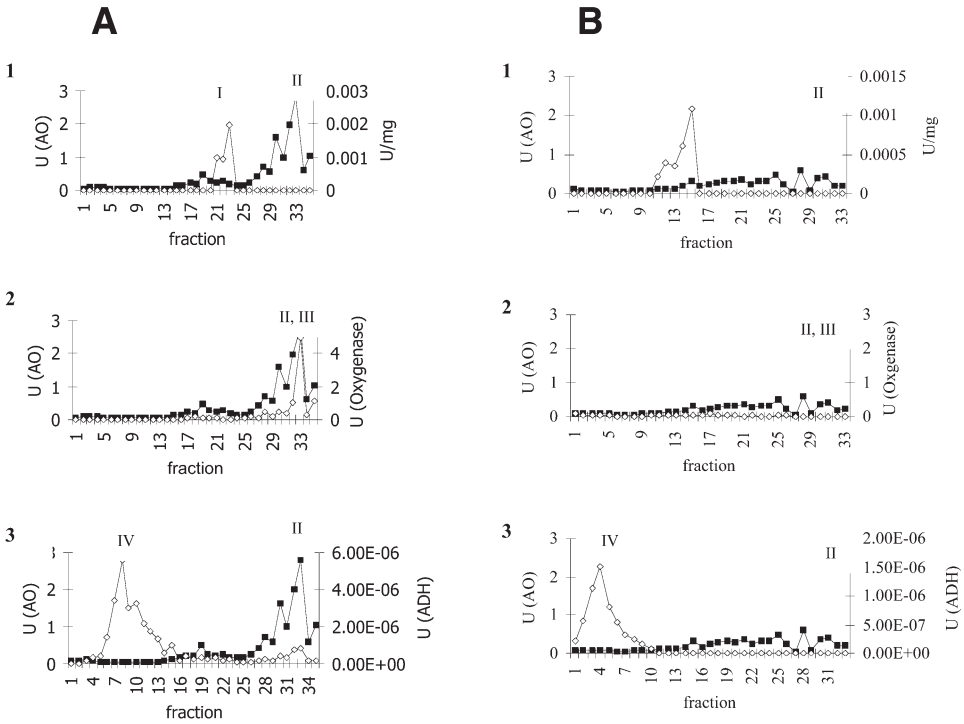

Fig. 3. Intracellular localization of AO activity. Mycelial cells grown in **(A)** decane or **(B)** glucose as the sole carbon source were gently homogenized as described in Materials and Methods, resuspended in a minimal volume of 0.05 *M* Tris-HCl buffer (pH 8.5), and then submitted to a sucrose isopycnic gradient (10–60%). Different enzymatic activities were assayed in each fraction of the gradient. (Panels 1) Distribution pattern of AO (■) and peroxidase (◊) using decanol as enzyme substrate, (Panels 2) distribution pattern of AO (■) and oxygenase (◊) using decane as enzyme substrate; (Panels 3) distribution pattern of AO (■) and alcohol dehydrogenase (◊) using ethanol as enzyme substrate. Roman numerals show the gradient fraction where each enzymatic activity was located: I, peroxidase; II, AO; III, oxygenase; IV, soluble ethanol dehydrogenase.

carbon sources, and after gentle cell homogenization samples were submitted to the isopycnic gradient. Comparative results of the samples from mycelia grown in decane and glucose can be seen in Fig. 3. In Fig. 3A, interestingly, the localization of AO activity by means of the density of the microbodies does not correspond to the localization of peroxidase, which is located in peroxisomes *(16)*. If one compares panels in Fig. 3A and Fig. 3B, it is clear that the AO activity and possibly also the microsomal bodies that could contain it are absent in the samples when the microorganism was grown in glucose as the sole carbon source. This result is consistent with our previous work, in which we reported that AO synthesis was induced by hydrocarbons and repressed by glucose *(15)*. Another important point in this experiment was the localization of the oxygenase activity in the sucrose gradients (Fig. 3, panels 2). In this case, the oxygenase activity was only

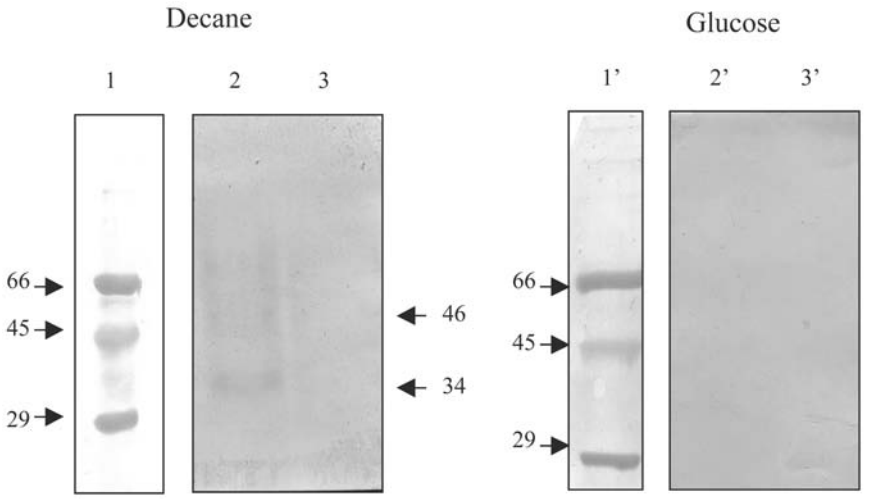

Fig. 4. Immunodetection of AO subunits in samples of the isopycnic gradient. *Lanes* 1 and 1, molecular weight markers; lanes 2 an 2′ samples of fractions 33 in decane or glucose (density = 1.25–1.26) of each isopicnic gradient; lanes 3 and 3′ samples of fractions 23 in decane and fraction 16 in glucose (density = 1.15–1.16) of each isopycnic gradient. All lanes were loaded with 20 μg of protein. Arrows indicate the bands detected by the homologous antibody.

detected in samples in which the carbon source for growth was decane panel 2 in Fig. 3A; in glucose samples, the activity was absent panel 2 in Fig. 3B. It is important to say that these results suggest that both enzymes, oxygenase and AO, are located in the same microsomal body, different from the peroxisomes. Panels 3 of Fig. 3 show the alcohol dehydrogenase activity, used here as a molecular marker of cytosolic fraction.

We wished to obtain more evidence to support the idea that AO and peroxidase activities are not in the same intracellular compartment, so we decided to utilize an immunochemical approach. We took aliquots from each enzymatic activity peak in the gradient: where the AO activity was detected in decane (Fig. 3A, panel 1, peak II), and the corresponding sample in the glucose experiment (Fig. 3B, panel 1) and the peaks where peroxidase activity was detected (Fig. 3A, Lane 1, peak I and Fig. 3B, Lane 2, peak I), all samples were submitted to SDS-PAGE and immunoblotting. The results can be seen in Fig. 4, where two different bands were detected by the antibody against AO: one of 46 kDa and the other of 34 kDa (lane 2, decane). The immunodetection pattern was consistent with the hypothesis that the AO activity is located in a specific microsomal body, different from the peroxisomes, because in the fraction where the peroxidase activity was located, no one AO band was recognized by the antibody (lane 3, decane). On the other hand, in the samples from the growth of strain YR-1 in glucose, no band was detected by the antibody. Table 2 gives the values of density measured in the different fractions of the sucrose isopycnic gradients

Table 2
Intracellular Distribution of Microsomal Bodies in Isopycnic Sucrose Gradients from Samples of YR-1 Strain Grown in Different Carbon Sources

Carbon source	Molecular marker	Density of vesicles[a]
Decane	AO	1.26
	Peroxidase	1.16
	Oxygenase	1.26
	Alcohol dehydrogenase	1.06
Decanol	AO	1.26
	Peroxidase	1.143
	Oxygenase	1.26
	Alcohol dehydrogenase	1.07
Methanol	AO	1.255
	Peroxidase	1.18
	Oxygenase	1.26
	Alcohol dehydrogenase	1.06
Hexadecane	AO	1.26
	Peroxidase	1.143
	Oxygenase	1.26
	Alcohol dehydrogenase	1.07
Glucose	AO	ND
	Peroxidase	1.16
	Oxygenase	n. d.
	Alcohol dehydrogenase	1.062

[a]ND, not detected.

made with samples from extracts in which the YR-1 strain was grown in different carbon sources. As can be seen in all cases, except in glucose, oxygenase and AO were of the same density whereas peroxidase was of a different density. This strongly suggests that the enzymes that participate in the hydrocarbon biodegradation in strain YR-1 could be located in a specific microsomal body different from the peroxisomes.

Discussion

Microbodies (peroxisomes, glyoxisomes, and glycosomes) represent a class of ubiquitous and important cell organelles that are characterized by a proteinaceous matrix surrounded by a single membrane, and their physiologic role is complex and variable. In particular, in yeasts, micro body-bound enzymes are crucial for the metabolism of specific growth substrates *(17)*. Intracellular localization of enzymes has been extensively studied, and one of the most important successes in this field is the particular localization of different enzymatic complexes or whole metabolic processes inclusive in specific intracellular compartments. However, some important metabolic processes such as hydrocarbon biodegradation are little understood mainly in filamentous fungi.

Some studies have demonstrated that AO activity is situated in the inner part of peroxisomes and that this enzyme participates in the aliphatic hydrocarbon biodegradation pathway *(17,18)*. In this context, our results contrast with this idea because they suggest that oxygenase and AO activities (which, respectively, catalyze the first and second enzymatic steps in the aliphatic hydrocarbon biodegradation pathway in YR-1 strain) are located in a specific microbody different from the peroxisome.

Also very important was our finding of two different AO activities. In Table 1, the results reveal the existence of the sAO described *(14)*, purified, and characterized *(15)* previously and a second activity associated with the MMF after a "drastic" ballistic cell homogenization procedure. These results were complemented by the zymogram in Fig. 1, which shows the results of cells grown in decane or methanol that underwent a "gentle" cell homogenization procedure. In this case the zymogram revealed that the AO activity remains in the sample application point of the gel; in other words, the AO does not run into the gel. This result suggests that AO activity could be associated with some kind of membrane that prevents its inclusion in the gel. To obtain evidence about the localization of the AO, principally sAO—because we think that this could be a particulate enzyme but that it must be in the lumen of some organelle and not necessarily included in any membrane matrix—first we developed an immunochemical approach using homologous antibodies against sAO obtained in previous work. The protein to be used as antigen after its blotting to a nitrocellulose membrane was obtained from cells grown in different carbon sources and purified by means of sucrose isopycnic gradients. As can be seen in Fig. 2A (cells grown in hexadecane and broken by the "drastic" procedure), two bands were detected (lanes 2 and 3), one of 46 kDa, the putative subunit of the sAO, and the other of 34 kDa, which could be part of the mAO because in lane 3 (the supernatant of the 104,500*g* centrifugation) the 34-kDa band was absent. Complementary to these results, the sucrose gradients experiments strongly suggest the existence of particular microbodies that contain both oxygenase and AO, and their density in the isopycnic gradients was quite different from the density shown by peroxisomes, microbodies that contain the peroxidase activity (Fig. 3). The results shown in Fig. 4 are consistent with the hypothesis of specific organelles for hydrocarbon biodegradation because the two bands of 46 and 34 kDa (Fig. 4, lane 2; decane) were detected only in the sample from the peak of the gradient where the AO activity was detected (Fig. 3A, panel, peak II). On the other hand, in the sample from the peak corresponding to the peroxidase (Fig. 4, lane 3; decane), no one band could be detected. Thus, the AO and, in this particular case, also the oxygenase activities appear to be in the same specific microbody, different from the peroxisome. These results were consistent independent of the carbon source present in the growth media.

Further experiments are necessary to obtain get evidence of the existence of these particular microbodies specific for hydrocarbon metabolism. We are currently initiating electron microscopy approaches to study the intracellular architecture of the YR-1 strain.

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