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Biosynthesis of peroxisomal enzymes in the methylotrophic yeast *Hansenula polymorpha*

(distinct polypeptides in methanol-grown cells/alcohol oxidase/catalase/mRNA translation/differences between translation products and mature enzymes)

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ABSTRACT **The dramatic expansion of the peroxisomal compartment known to occur in the methanol-utilizing yeast *Hansenula polymorpha* on transfer from glucose- to methanol-containing media was shown to be accompanied by the synthesis of at least six major polypeptides that dominate the polypeptide pattern of total cell extracts analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Two of these polypeptides have been identified by immunochemical methods as the monomers of the peroxisomal enzymes alcohol oxidase and catalase. We have studied the biosynthesis of these two peroxisomal enzymes, both by *in vitro* translation and by *in vivo* labeling experiments. By the criterion of mobility in $NaDodSO_4$/polyacrylamide gel electrophoresis, the *in vitro*- and *in vivo*-synthesized monomers were indistinguishable from each other, both in the case of alcohol oxidase and in that of catalase. Thus, neither of these peroxisomal enzymes appear to be synthesized as larger precursors. However, further analysis of *in vitro*-synthesized versus mature peroxisomal alcohol oxidase showed that the *in vitro*-synthesized form sedimented as a 5S monomer and not, like the mature peroxisomal enzyme, as a 20S octamer. Moreover, the *in vitro*-synthesized form was highly susceptible to trypsin digestion whereas the mature 20S octamer appeared to be resistant.**

In the present study, we have used the methylotrophic yeast *Hansenula polymorpha* as a model system to study the biosynthesis of peroxisomal enzymes. In most cells, peroxisomal enzymes amount to less than 1% of total cellular proteins. The biosynthesis of peroxisomal enzymes and the biogenesis of peroxisomes as organelles is therefore difficult to study. *H. polymorpha* cells offer a distinct advantage in this respect. When these cells are transferred from glucose- to methanol-containing media, the synthesis of four enzymes involved in methanol metabolism is derepressed (ref. 1; for review, see ref. 2). Although only two of the four enzymes, alcohol oxidase and catalase, are located in the peroxisomes (the other two, formaldehyde and formate dehydrogenase, are located in the cytoplasm), the peroxisomal compartment undergoes a striking expansion. A single small peroxisome in glucose-grown cells develops in methanol-grown cells into a large peroxisome from which other peroxisomes appear to separate until the peroxisomal compartment occupies much of the cellular space (2).

In this report, we compare the changes in polypeptide patterns of extracts of *H. polymorpha* grown in glucose- and methanol-containing media. We also compare the cell-free translation products of alcohol oxidase and catalase with the mature peroxisomal enzymes.

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MATERIALS AND METHODS

The following procedures used in this study have been described elsewhere: extraction of total RNA by using $NaDodSO_4$/phenol/chloroform/isoamyl alcohol (3), translation of total RNA in a staphylococcal nuclease-treated rabbit reticulocyte lysate (4), immunoprecipitation of $NaDodSO_4$-denatured polypeptides (5), $NaDodSO_4$/polyacrylamide gel electrophoresis (6) and subsequent radioautography or fluorography (7), and blot transfer of polypeptides from gels to nitrocellulose and antibody identification of antigen on nitrocellulose blots (8).

Cultivation and Labeling of Cells. *H. polymorpha*, a methylotrophic yeast, was obtained from the American Type Culture Collection. Strain ATCC 26012 was used in all experiments. Cells were grown at 37°C to midexponential phase ($\approx 8 \times 10^7$ cells per ml; optical density at 600 nm ≈ 1.0) in complete medium (9) (for RNA extraction only) or in sulfate-free semisynthetic medium (10) (for all other experiments). These media were supplemented either with 0.5% glucose or 0.5% methanol. For continuous labeling of cells, growth was for at least 10 generations in the presence of 4 mCi of carrier-free $Na_2{}^{35}SO_4$ per liter (1 Ci = 3.7×10^{10} Bq).

Preparation of Cell Extract. A total extract was prepared by a modification of the glass bead procedure of Lang *et al.* (11). After harvesting, cells were washed twice in buffer A [150 mM NaCl/2 mM $Mg(OAc)_2$/10 mM Tris·HCl, pH 7.4] and then suspended (0.4 g of cells per ml) in buffer A containing a mixture of protease inhibitors (12). Sterile glass beads (0.45- to 0.50-mm diameter; 3–4 g per ml of suspension) were added and the mixture was spun in a Vortex for 3–4 min at 4°C. The resulting homogenate was carefully removed using a Pasteur pipette with a drawn-out tip. Unbroken cells were removed by centrifugation for 15 sec at 12,000 × *g* in an Eppendorf Microfuge. The resulting supernatant is referred to as total extract. For analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis, total extracts were incubated for 5 min in a boiling water bath in the presence of $NaDodSO_4$ (3% final concentration). For analysis on sucrose density gradients (see Fig. 4), total extracts were briefly sonicated (four 1-sec pulses in a Branson sonicator).

Preparation of *H. polymorpha* Cell-Free Translation. A 30,000 × *g* supernatant, referred to as S-30, was prepared from methanol-grown *H. polymorpha* exactly as described for *Saccharomyces cerevisiae* (13). The S-30 was then incubated with [^{35}S]methionine (1 mCi/ml) as described (13).

Preparation of Antisera. Commercially available alcohol oxidase of *Candida boidinii* was more than 90% pure as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis analysis (see

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Fig. 2A, lane 3). The 75,000-dalton band was excised from the gel and the homogenized material was used for immunization of rabbits by standard procedures. Our source for catalase was a 30–40% ammonium sulfate precipitate of an *H. polymorpha* extract (14) provided to us by J. P. van Dijken (Delft, The Netherlands). Catalase activity of this extract was further purified by DEAE-cellulose chromotography (14). By $NaDodSO_4$/polyacrylamide gel electrophoresis analysis of the active fractions, one of the major bands [see Fig. 2A (arrow in lane 4)] was identified as catalase on the basis of its apparent M_r of 56,000. This band was excised from the gel and the homogenized material was used for immunization of rabbits.

Source of Materials. Alcohol oxidase from *C. boidinii* was from Sigma. Carrier-free $Na_2{}^{35}SO_4$ (1.0 Ci/mmol) and [^{35}S]methionine (1,000 Ci/mmol) were from New England Nuclear. Glass beads were from B. Braun (Melsungen, F.R.G.).

RESULTS

Synthesis of Several Distinct Polypeptides After Derepression by Methanol. To compare the polypeptide patterns of *H. polymorpha* grown in glucose- or methanol-containing media, cells were harvested at various cell densities (Fig. 1) and total cell extracts were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Whereas cells grown in glucose-containing media showed no significant changes in their polypeptide pattern (lanes 1–4), there were striking changes after growth in methanol-containing media (lanes 5–8). Six polypeptides dominated the differential pattern. One of them, P42, appeared very early—i.e., after 1 to 2 doubling times (lane 5). The other five polypeptides appeared more or less together—i.e., after 5 to 6 doubling times (lanes 7 and 8).

Two of the six polypeptides have been identified: P75 is the monomer of alcohol oxidase and P56 is the monomer of catalase (Fig. 2). Two other polypeptides can be tentatively identified. Based on their mobility in $NaDodSO_4$/polyacrylamide gel electrophoresis and their known molecular weights, P39 and P36 appear to be the monomers of formaldehyde dehydrogenase and formate dehydrogenase (15), respectively. Two polypeptides (P78 and P42) remain unidentified.

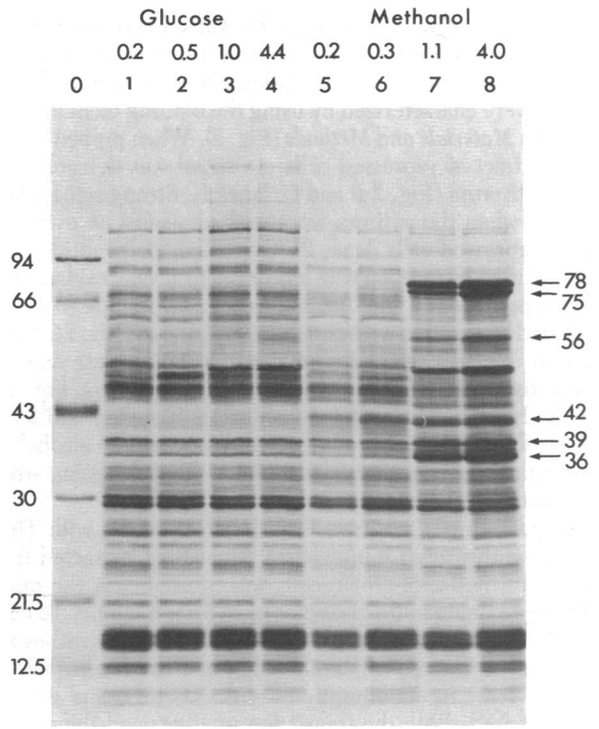

FIG. 1. Comparison of polypeptide patterns of total extracts of *H. polymorpha* grown in media containing glucose (lanes 1–4) or methanol (lanes 5–8). Aliquots of cells previously grown in glucose were inoculated in fresh medium containing either glucose or methanol at an OD_{600} of 0.05/ml. Cells were harvested thereafter at the absorbance at 600 nm indicated above each lane. Seven microliters of total extract (corresponding to about 3 mg of cells) was reduced, alkylated, and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis on 10–15% acrylamide gradient gels. The gels were stained with Coomassie blue. The following molecular weight markers (indicated $\times 10^{-3}$ on the left) were coelectrophoresed in lane 0: rabbit muscle phosphorylase A (94,000), bovine serum albumin (66,000), chicken ovalbumin (43,000), bovine carbonic anhydrase B (30,000), soybean trypsin inhibitor (21,500), and horse heart cytochrome *c* (12,500). Numbers on arrows to the right of lane 8 give the masses (in kilodaltons) of major polypeptides appearing in methanol derepressed cells.

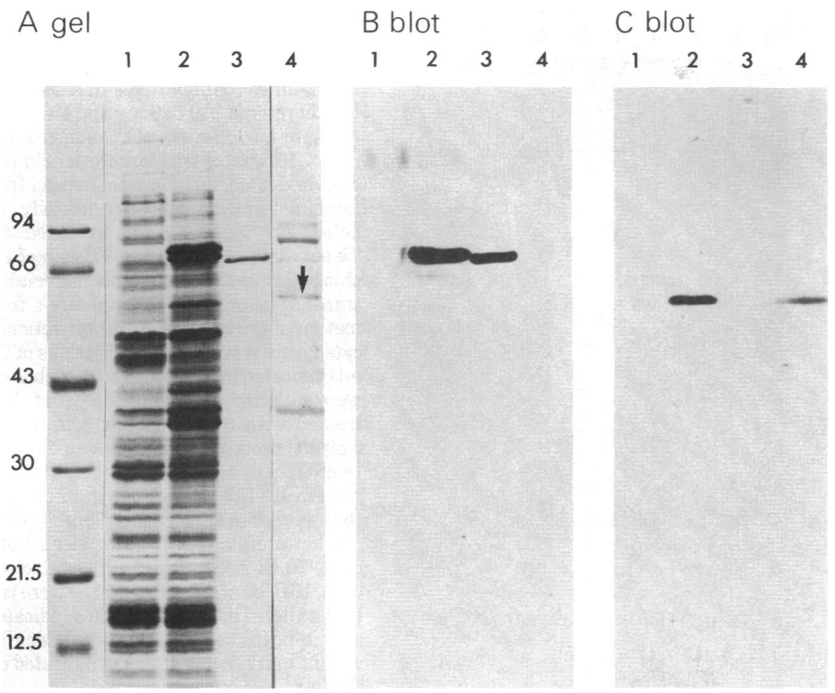

FIG. 2. Immunochemical characterization of total extracts from glucose- or methanol-grown *H. polymorpha*. Total extracts from glucose-grown cells (lanes 1) or from methanol-grown cells (lanes 2), alcohol oxidase of *C. boidinii* (lanes 3), and a partially purified catalase preparation from *H. polymorpha* (lanes 4) were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by blot transfer to nitrocellulose. Three parallel gel segments were prepared: one was not blotted but stained with Coomassie blue (*A*). The other two were blotted and probed with antiserum to alcohol oxidase (*B*) or antiserum to catalase (*C*). Molecular weight markers are as in Fig. 1. The arrow in lane 4 of *A* indicates catalase monomer. Note that the commercially obtained alcohol oxidase of *C. boidinii* migrated slightly faster than the alcohol oxidase in *H. polymorpha* extract (compare lanes 2 and 3 of *B*).

Characterization of Monospecific Antisera Against Alcohol Oxidase and Catalase. Monospecific antisera raised in rabbits against purified and $NaDodSO_4$-denatured alcohol oxidase or catalase were characterized by using the blotting technique described in *Materials and Methods* (Fig. 2). When probed against a total extract of repressed cells, no signal was detected with either antiserum (Fig. 2 *B* and *C*, lanes 1). Strong signals were detected when the antisera were probed against an extract of fully derepressed cells (lanes 2). One major polypeptide (P75) was identified by reaction with antiserum to alcohol oxidase (Fig. 2*B*, compare lanes 2 and 3) and another polypeptide (P56) was identified by reaction with antiserum to catalase (Fig. 2*C*, lanes 2 and 4). No crossreactivity between alcohol oxidase and catalase antisera could be detected (Fig. 2*B*, lane 4, vs. Fig. 2*C*, lane 3). In agreement with the report of Eggeling and Sahm (16), we found a strong crossreactivity between the alcohol oxidase from *H. polymorpha* and that from *C. boidinii* (against which our antiserum was raised).

Comparison of Cell-Free Translation Products with Their *in Vivo*-Synthesized Counterparts. Total RNA extracted from glucose- or methanol-grown cells was translated in the rabbit reticulocyte lysate in the presence of [^{35}S]methionine. To compare the translation products with their *in vivo*-synthesized counterparts, glucose- or methanol-grown cells were labeled during at least 10 doubling times with $Na_2{}^{35}SO_4$ and extracts were prepared. Both the translation products and the cell extracts were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and subsequent fluorography (Fig. 3, lanes 1–4). In the case of methanol-grown cells, we also analyzed immunoprecipitates of the translation products and of the total extracts using antialcohol oxidase serum (lanes 6 and 7), anticatalase serum (lanes 9 and 10), or the corresponding preimmune sera (lanes 5 and 8).

Differentially present among the total translation products of RNA (lane 2) or the total *in vivo*-labeled products (lane 4) from derepressed cells were polypeptides of 78,000 and 75,000 daltons. The *in vitro*-synthesized 75,000-dalton polypeptide was immunoprecipitated by antiserum to alcohol oxidase (lane 6)

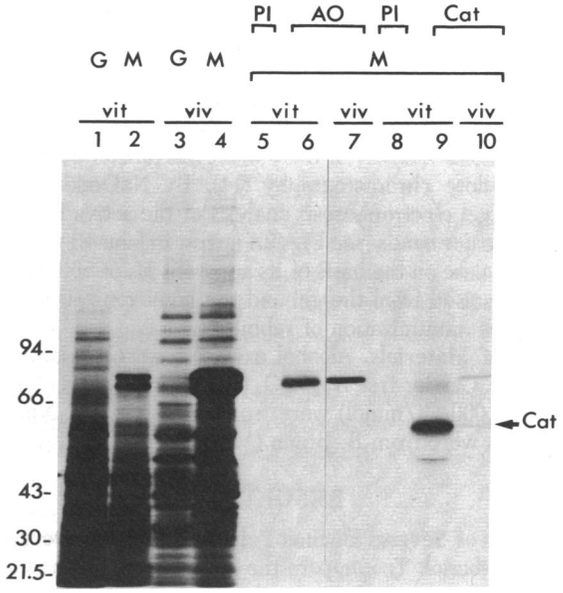

FIG. 3. Comparison of cell-free translation products (vit) with *in vivo*-labeled products (viv) of glucose (G)- or methanol (M)-grown *H. polymorpha*. Total products (lanes 1–4) or immunoprecipitates (lanes 5–10) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and the gels were analyzed by fluorography. PI, AO, and Cat, immunoprecipitates obtained with preimmune serum, alcohol oxidase antiserum, or catalase antiserum, respectively. Molecular weight markers are as in Fig. 1. The catalase band is indicated by an arrow. Bands above the catalase band in lanes 9 and 10 are contaminants; the band below the catalase band in lane 9 is an artifact due to the heavy chain of IgG.

and it showed the same electrophoretic mobility as its *in vivo*-synthesized counterpart (lane 7). Likewise, no difference in mobility could be detected between the corresponding forms of catalase (lanes 9 and 10).

Further Characterization of *in Vitro*-Synthesized Alcohol Oxidase. In the peroxisomal compartment, alcohol oxidase is a

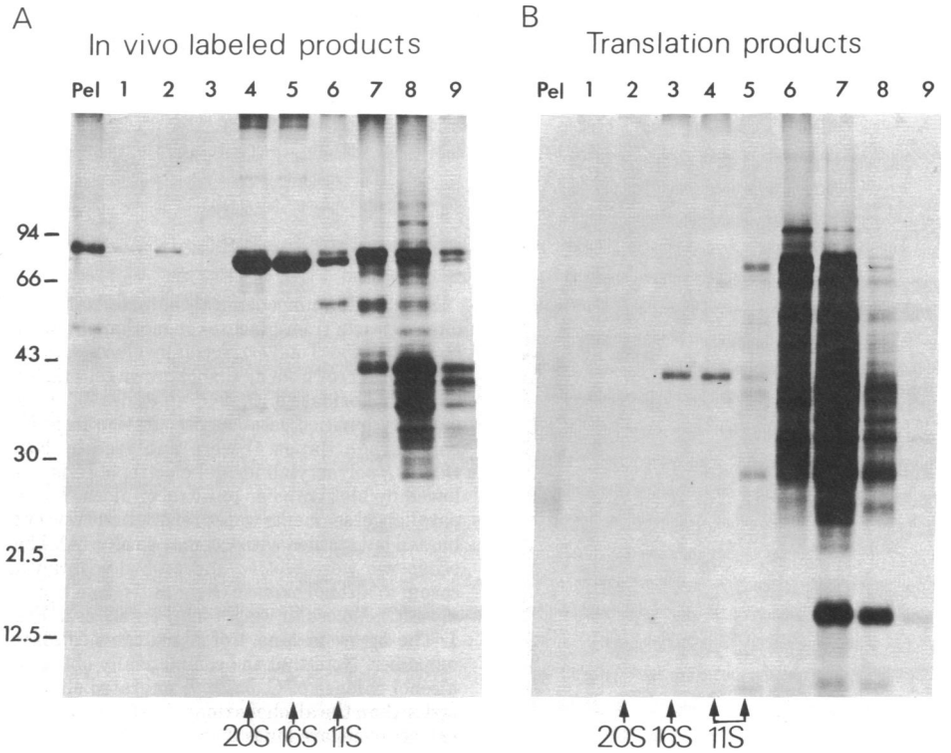

FIG. 4. Comparison of sedimentation rates of *in vivo*-labeled products (*A*) and cell-free translation products (*B*). Gradients of 10–30% sucrose in buffer A containing a mixture of protease inhibitors and gelatin at 10 μg/ml were centrifuged in 5.2-ml centrifuge tubes in a swinging bucket rotor at 210,000 × *g* and 4°C for 5.5 hr (*A*) or for 11 hr (*B*). (*A*) Ten microliters (1.5×10^6 cpm of trichloroacetic acid-precipitable material) of a total extract from methanol-grown cells continuously labeled with $Na_2{}^{35}SO_4$ was analyzed. (*B*) Twenty microliters (1.5×10^6 cpm of trichloroacetic acid-precipitable material) of translation products of total RNA from methanol-grown cells in rabbit reticulocyte lysate was analyzed. Fractions of 0.5 ml (indicated in numbers above each lane) were collected, including the pellet (Pel) treated with trichloroacetic acid to precipitate protein, and processed for $NaDodSO_4$/polyacrylamide gel electrophoresis in 10–15% acrylamide gradient gels that were then analyzed by fluorography. Marker proteins (*C. boidinii* alcohol oxidase, 20 S; *Escherichia coli* β-galactosidase, 16 S; bovine catalase, 11 S) were run in parallel. Their positions are indicated at the bottoms of the gels. Molecular weight markers ($\times 10^{-3}$) are indicated on the left.

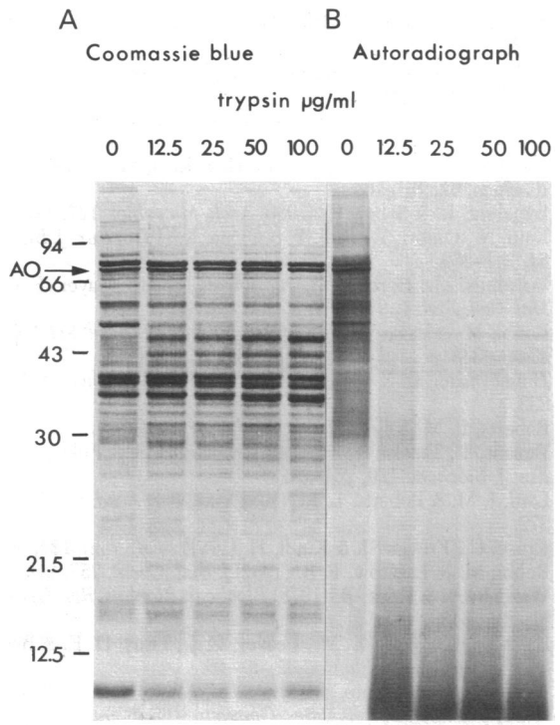

FIG. 5. Differential susceptibility of newly synthesized and mature alcohol oxidase to trypsin digestion. An S-30 fraction from methanol-grown *H. polymorpha* was incubated with [^{35}S]methionine. Aliquots of 5 μl were incubated with trypsin for 1 hr at 0°C, at the final concentrations indicated above the lanes. Trypsin digestion was terminated by addition of Trasylol (1,000 units/ml) and the mixture was immediately analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. After staining with Coomassie blue, the slab gel was autoradiographed. Molecular weight markers are as in Fig. 1. The arrow on the left points to the alcohol oxidase (AO) band.

20S octamer consisting of eight identical subunits with each subunit containing one noncovalently bound FAD as prosthetic group (17). The 20S octamers appear to be the main structural component of the crystalloids found within the peroxisomal compartment (18). To compare the sedimentation behavior of the *in vitro*-synthesized form of alcohol oxidase with the form of alcohol oxidase present in extracts of $Na_2{}^{35}SO_4$-labeled methanol-grown cells, we carried out sucrose density gradient centrifugation. As expected, the alcohol oxidase in the extract prepared from $Na_2{}^{35}SO_4$-labeled derepressed cells sedimented almost exclusively in the 20S region of the gradient (Fig. 4*A*). In contrast, alcohol oxidase synthesized by translation of mRNA in a rabbit reticulocyte lysate sedimented as a 5S monomer (Fig. 4*B*).

Another distinction between the *in vitro*-synthesized form of alcohol oxidase and its peroxisomal counterpart is their differential sensitivity to trypsin. For this experiment, alcohol oxidase was synthesized in an S-30 extract of derepressed cells. This extract contains both the labeled newly synthesized alcohol oxidase (resulting from polysome readout in the presence of [^{35}S]methionine) and the unlabeled 20S form derived from the fragmented peroxisomal compartment (Fig. 4*A*). Whereas the newly synthesized alcohol oxidase was rapidly degraded (Fig. 5*B*), the 20S form appeared to be resistant to digestion, even at high trypsin concentration (Fig. 5*A*).

DISCUSSION

We have shown that the transfer of *H. polymorpha* from glucose- to methanol-containing media resulted in the synthesis of at least six major polypeptides. Except for one polypeptide (M_r, 42,000), the synthesis of these polypeptides occurred after a lag period of several hours (corresponding to five or six cell divisions) after transfer from glucose- to methanol-containing media. Using monospecific antibodies, we have identified two of these polypeptides as the monomers of alcohol oxidase (M_r, 75,000) and of catalase (M_r, 56,000). Two of the other polypeptides (M_r, 39,000, and M_r, 36,000) can be tentatively identified on the basis of their molecular weights as formaldehyde dehydrogenase and formate dehydrogenase, respectively, both cytoplasmic enzymes (2, 15). Two of the major polypeptides (M_r, 78,000, and M_r, 42,000) are of unknown function. One of them (M_r, 78,000) behaved like a peroxisomal component: in cell fractionation experiments of gently lysed spheroplasts, this protein cofractionated with alcohol oxidase (data not shown).

Our finding that alcohol oxidase and catalase are among the major polypeptides in methanol-grown cells facilitated studies on their biosynthesis. Alcohol oxidase was readily detectable, without immunoprecipitation as an enrichment procedure, among the total polypeptides of the cell extract (Fig. 1, lane 8) as well as among the products obtained after translation of RNA in a rabbit reticulocyte lysate system (Fig. 3, lane 2). Comparison of the *in vitro*-synthesized alcohol oxidase and the mature peroxisomal enzyme in NaDodSO$_4$/polyacrylamide gels showed no difference in electrophoretic mobility, suggesting that alcohol oxidase is not synthesized as a larger precursor. Similar findings were made for catalase. It remains to be investigated, however, whether the *in vitro*-synthesized forms constitute the primary translation products or some modification thereof (e.g., proteolytically modified at either the NH_2 or COOH terminus). Although several peroxisomal enzymes have now been reported to be synthesized in cell-free systems, not as larger precursors (5, 19–25), minor proteolytic modifications have not yet been ruled out. In the case of rat liver catalase, Robbi and Lazarow (25) have presented data that argue strongly against proteolytic modification of the primary translation product in the cell-free translation system. It should be noted, however, that several peroxisomal enzymes have been reported to be synthesized as larger precursors (26–28), suggesting that they contain a cleavable signal sequence addressed to the postulated peroxisomal translocation machinery (5, 29). It is likely that those peroxisomal enzymes that are not synthesized as larger precursors nevertheless contain the equivalent of a signal sequence that is not cleaved. This situation is analogous to that encountered with polypeptides that have to be translocated across the membranes of the rough endoplasmic reticulum, mitochondria, or chloroplasts. Although in all these cases uncleaved signal sequences do occur, they constitute a small percentage of the examples so far studied. In the case of peroxisomal enzymes, uncleaved signal sequences appear to be more the rule than the exception.

We have attempted to develop an *in vitro* translocation system by supplementing the cell-free translation system with a peroxisome fraction isolated from methanol-grown *H. polymorpha* and then assaying for protease resistance of alcohol oxidase as a criterion for successful translocation. Other investigators have reported success in this respect (20, 24) with analogous material from other sources. So far, however, our attempts to develop such a system have failed.

In the course of comparing the *in vitro* translation product of alcohol oxidase with the mature peroxisomal enzyme, we noted two differences between them. Unlike the mature peroxisomal enzyme, which is an octamer sedimenting at 20 S (17), the *in vitro* translation product sedimented at 5 S and therefore appeared to be a monomer. Furthermore, the *in vitro* translation product was very sensitive to trypsin digestion whereas the ma-

ture 20S octamer appeared to be resistant to such digestion, even to high trypsin concentrations. Taken together and by analogy to the available data on catalase (30, 31), our results suggest that alcohol oxidase exists in a monomeric form outside of the peroxisome. Aggregation to an octamer (in the case of catalase, a tetramer) and the noncovalent attachment of a FAD moiety as prosthetic group (in the case of catalase, heme) occur after translocation into peroxisomes.

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