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Dihydroxyacetone synthase is localized in the peroxisomal matrix of methanol-grown *Hansenula polymorpha*

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Abstract. The subcellular localization of dihydroxyacetone synthase (DHAS) in the methylotrophic yeast *Hansenula polymorpha* was studied by various biochemical and immunocytochemical methods. After cell fractionation involving differential and sucrose gradient centrifugation of protoplast homogenates prepared from methanol-grown cells, DHAS cosedimented with the peroxisomal enzymes alcohol oxidase and catalase. Electron microscopy of this fraction showed that it contained mainly intact peroxisomes, whereas SDS-polyacrylamide gel electrophoresis revealed two major protein bands (75 and 78 kDa) which were identified as alcohol oxidase and DHAS, respectively. The localization of DHAS in peroxisomes was further established by immunocytochemistry. After immuno-gold staining carried out on ultrathin sections of methanol-grown *H. polymorpha* using DHAS-specific antibodies, labelling was confined to the peroxisomal matrix.

Key words: *Hansenula polymorpha* — Peroxisomes — Methanol — Dihydroxyacetone synthase — Cell fractionation — Immunocytochemistry

During growth of methylotrophic yeasts on methanol peroxisomes play an indispensable role since they exclusively harbour the two key enzymes of the initial metabolism of the substrate, namely alcohol oxidase and catalase (van Dijken et al. 1975; Fukui et al. 1975; Roggenkamp et al. 1975; Veenhuis et al. 1976). The C₁ reaction product of the alcohol oxidase, formaldehyde, is subsequently either dissimilated to carbon dioxide via two cytosolic dehydrogenases (Fukui et al. 1975; Tanaka et al. 1976; Jenkins et al. 1985) or assimilated by way of the xylulose monophosphate (XuMP) cycle (van Dijken et al. 1978; Veenhuis et al. 1983).

Little is known about the subcellular localization of the various enzymes of this XuMP cycle. On the basis of fractionation experiments, Jenkins et al. (1985) suggested that in methanol-grown *Candida boidinii* the key enzyme of this cycle dihydroxyacetone synthase (DHAS) — which catalyses the transfer of a glycolaldehyde group from xylulose 5-phosphate to formaldehyde and forms glyceraldehyde 3-phosphate and dihydroxyacetone — is located in

mitochondria. However, a number of other observations have indicated that DHAS may in fact be localized in the peroxisomes of methylotrophic yeasts. For instance, Goodman et al. (1984) showed that in a peroxisomal fraction isolated from methanol-grown *C. boidinii*, two major polypeptides were present that differed by approximately 3 kDa in subunit molecular weight. Polypeptide bands with similar molecular weight have been observed in *Hansenula polymorpha* after adaptation to growth on methanol (Roa and Blobel 1983). In both organisms one of these polypeptides, namely that with the smaller molecular weight, was identified as alcohol oxidase (Roa and Blobel 1983; Goodman et al. 1984). The other polypeptide was not identified, but it behaved like a peroxisomal component (cf. Roa and Blobel 1983) and might represent DHAS since its molecular weight was identical to that of the DHAS of *H. polymorpha* (Roggenkamp et al. 1984).

These observations, together with the fact that a possible localization of DHAS in peroxisomes of methanol-grown yeasts has important regulatory consequences, prompted us to study the subcellular localization of this enzyme. The results of this study are presented in this paper.

Materials and methods

Organism and cultivation. *Hansenula polymorpha* de Morais et Maya CBS 4732 was used in all experiments. The organism was grown in a batch fermenter at 37°C in a mineral medium containing per litre: (NH₄)₂SO₄ 2.5 g; KH₂PO₄ 1 g; MgSO₄ · 7H₂O 0.2 g; yeast extract 0.5 g; trace elements according to Vishniac and Santer (1957) 0.2 ml; biotin 5 µg; thiamine-HCl 300 µg. Methanol (0.5% v/v) was added as a carbon and energy source. The pH of the culture was maintained at pH 5.0 with 1 M NaOH. The fermenter cultures were inoculated with cells grown on glucose as described previously (Veenhuis et al. 1979) and were harvested in the exponential growth phase (OD₆₆₃ = 1.8).

Transfer experiments. Glucose was added to cultures growing exponentially on methanol (0.5% v/v) to a final concentration of 1.0% (w/v).

Cell fractionation. Cells were harvested by centrifuging and washed in 50 mM potassium phosphate buffer pH 7.5 containing 1 mM MgCl₂ and 1 mM EDTA (buffer A), resuspended in buffer A plus 0.2 M 2-mercaptoethanol and 3 M sorbitol and incubated at 35°C for 10 min. After washing twice with buffer A plus 3 M sorbitol to remove the

Abbreviations. MES, 2-(N-morpholino)ethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; TPP, thiamin pyrophosphate; DHAS, dihydroxyacetone synthase; GSH, reduced glutathione

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mercaptoethanol, the cells were resuspended in the same buffer (0.06 g wet weight/ml). Then the cell wall digesting enzyme Zymolyase 20,000 was added to a final concentration of 1 mg/ml and the mixture was incubated for 3 h at 35°C. All further steps were carried out in 5 mM MES-buffer pH 5.5 containing 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 0.5 mM TPP (buffer B) in order to stabilize DHAS activity (Waites and Quayle 1983). After cell wall digestion, the protoplasts were sedimented (10 min 10,000 × g) and washed twice with buffer B plus 3 M sorbitol, then dialysed against buffer B plus 1 M sorbitol for 1 h at 4°C and finally homogenized in a Potter Elvehjem homogenizer (Potter 1955) by applying 10 strokes at 200 rpm. The homogenate was differentially centrifuged to remove cell debris and large cell organelles by centrifuging at 6,500 × g, 9,000 × g and 10,000 × g for 10 min and at 30,000 × g for 20 min. The pellet obtained at 30,000 × g was resuspended in a small volume of buffer B containing 35% sucrose. Subsequently 1 ml of this suspension was layered on top of a discontinuous sucrose gradient, composed of buffer B with the following sucrose concentrations (w/w): 65% (6 ml), 48% (2.5 ml), 46% (2.5 ml), 44% (7.5 ml), 42% (4 ml), 40% (5 ml). After the addition of an overlay containing 25% sucrose, the gradients were centrifuged in a Sorvall SS90 vertical rotor at 34,500 × g · av for 3 h at 4°C. The gradients were harvested by removing samples of 1.2 ml from the top. These samples were prepared for electron microscopy and tested for enzyme activities.

Enzyme assays. Activities of alcohol oxidase (EC 1.1.3.13) and formaldehyde dehydrogenase (EC 1.2.1.1) were assayed as described by van Dijken et al. (1976a), catalase (EC 1.11.1.6) by the spectrophotometric method of Lück (1963), dihydroxyacetone synthase according to Waites and Quayle (1981) and citrate synthase (EC 4.1.3.7) as described by Srere (1969). Cytochrome *c* oxidase (EC 1.9.3.1) activity was determined by following the oxidation of reduced cytochrome *c* at 550 nm in 50 mM potassium phosphate buffer pH 7.0 containing 40 µM reduced cytochrome *c*. Cytochrome *c* was reduced with sodium dithionite which was removed by Sephadex G25 chromatography. Alcohol oxidase activity is expressed as µmol O₂ consumed · min⁻¹ · mg protein⁻¹, formaldehyde dehydrogenase activity as µmol NADH formed · min⁻¹ · mg protein⁻¹, catalase activity as ΔE₂₄₀ · min⁻¹ · mg protein⁻¹, dihydroxyacetone synthase activity as µmol formaldehyde consumed · min⁻¹ · mg protein⁻¹, citrate synthase activity as µmol citrate formed min⁻¹ · mg protein⁻¹ and cytochrome *c* oxidase activity as µmol reduced cytochrome *c* oxidized · min⁻¹ · mg protein⁻¹. Protein concentrations were measured as described by Bradford (1976) using bovine serum albumin as a standard. Sucrose concentrations were determined by measuring the refractory index.

Enzyme purification and preparation of antisera. Dihydroxyacetone synthase was purified according to the method of Waites and Quayle (1983), and subsequently subjected to chromatography on DEAE-Sephacel using a continuous gradient of 0–500 mM ammonium sulphate. Final purification by preparative gel electrophoresis and the subsequent preparation of antiserum was performed as described by Vos-Scheperkeuter (1983). Alcohol oxidase was purified by ammonium sulfate precipitation. The fraction precipitating between 40 and 50% saturation contained most of the activity and was subjected to Sephacryl S300 gel filtration

using a 100 mM potassium phosphate buffer pH 7.5 followed by chromatography on DEAE-Sephacel using a continuous gradient of 0–500 mM ammonium sulphate. After rechromatography on Sephacryl S300 and dialysis against 10 mM potassium phosphate buffer pH 7.5, hydroxyapatite chromatography was performed. Protein was eluted from this column with a continuous 10–400 mM potassium phosphate gradient pH 7.5. The purified protein was used for the preparation of antiserum. The quality of the antisera was tested in Ouchterlony double diffusion tests. Alcohol oxidase antiserum showed a single precipitation band with purified alcohol oxidase and with cell-free extracts from methanol-grown cells. No visible immunoprecipitate was formed with purified dihydroxyacetone synthase. Similar results were obtained in Ouchterlony tests with dihydroxyacetone synthase antiserum.

SDS-polyacrylamide gel electrophoresis, using 10% gels, was carried out as described by Laemmli (1970). Silver staining of gels was performed as described by Wray et al. (1981).

Electron microscopy and immunocytochemistry. Intact cells were fixed with 3% glutaraldehyde in 0.1 M potassium phosphate buffer pH 7.0 for 45 min at 0°C, dehydrated in a graded ethanol series and embedded in Lowicryl K4M. Polymerization was at –35°C with UV light. Spheroplasts and subcellular fractions were fixed in glutaraldehyde/OsO₄/K₂Cr₂O₇ and embedded in Epon by methods described previously (Veenhuis et al. 1978). Protein A-gold labelling of alcohol oxidase and dihydroxyacetone synthase was performed on ultrathin sections of K4M-embedded cells by the method of Slot and Geuze (1984). Gold particles of 11 nm were prepared by the citrate method of Frens (1973), 3 nm gold particles by using white phosphorus (Roth 1982).

Results

Cell fractionation studies

To investigate the subcellular localization of DHAS in methanol-grown *Hansenula polymorpha*, cell fractionation studies were performed. After homogenization of protoplasts and various differential centrifugation steps to remove cell debris and large organelles (see Methods) the 30,000 × g pellet was separated on a discontinuous sucrose gradient. The recovery of alcohol oxidase and DHAS activities in the 30,000 × g pellet was low, namely approximately 5% of the activities initially present in the crude homogenates. Bulk of the activities of both enzymes were found in the low-speed pellets obtained during differential centrifugation. This was mainly due to inadequate formation of protoplasts and incomplete disruption of those that were formed as was indicated by electron microscopy of these pellets. So far attempts to improve recovery in the high speed pellet have failed.

After sucrose gradient centrifugation of the 30,000 × g pellet, 2 distinct bands were observed at approximately 45% and 52% sucrose. Typical patterns of protein and enzyme activities recovered from these gradients are shown in Fig. 1. As is evident from this figure, DHAS cosedimented in fractions 17–20 together with the peroxisomal enzymes alcohol oxidase and catalase in a small band of protein at approximately 52% sucrose. These peroxisomal enzymes were clearly separated from the mitochondrial marker

Fig. 1 A, B. Distribution of mitochondrial and peroxisomal enzymes after discontinuous sucrose gradient centrifugation of a fraction enriched in peroxisomes. This fraction was obtained by differential centrifugation of homogenized *Hansenula polymorpha* protoplasts. Cells were grown in batch cultures with methanol and harvested in the exponential growth phase ($OD_{663} = 1.8$). ($\blacktriangle$) Sucrose concentration; ($\triangle$) protein concentration; ($\square$) alcohol oxidase; ($\circ$) catalase; ($\blacksquare$) dihydroxyacetone synthase; ($\bullet$) cytochrome *c* oxidase. Alcohol oxidase is expressed as $\mu\text{mol O}_2$ consumed $\cdot \text{min}^{-1} \cdot \text{ml}^{-1}$, catalase as $\Delta E_{240} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$, DHAS as μmol formaldehyde consumed $\cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ and cytochrome *c* oxidase as μmol cytochrome *c* oxidized $\cdot \text{min}^{-1} \cdot \text{ml}^{-1}$.

enzyme cytochrome *c* oxidase which sedimented at a lower density and was present in a broader band of protein at approximately 45% sucrose. The peak fraction of the peroxisomal enzymes (fraction 19) was subjected to SDS-polyacrylamide gel electrophoresis in order to investigate its protein composition. As is shown in Fig. 2, this fraction is mainly composed of 2 proteins of apparent masses of 75 and 78 kDa, which were tentatively identified as alcohol oxidase and DHAS, respectively because of their position on the gel.

The peroxisomal nature of DHAS was also investigated after a shift of cells from methanol- to glucose-containing media. These experiments were based on the finding that peroxisomes — but not the mitochondria — in methanol-grown cells of *H. polymorpha* are rapidly degraded under glucose-excess conditions (Veenhuis et al. 1983). Since this process is associated with proteolytic degradation of the peroxisomal matrix proteins, a comparison of the kinetics of the disappearance of enzyme activities under these

Fig. 2. Protein composition of purified peroxisomes. Fraction 19 of the sucrose gradient (lane 5), purified alcohol oxidase (lane 3) and DHAS protein (lane 2) and extracts of methanol-grown *H. polymorpha* (lane 4) were subjected to SDS-polyacrylamide gel electrophoresis and silver stained. The following molecular weight markers (indicated on the left) were co-electrophoresed in lane 1: chymotrypsinogen A (25 kDa); aldolase (39.5 kDa); ovalbumin (44 kDa); bovine serum albumin (68 kDa).

conditions also allows to establish their subcellular localization. The results, shown in Fig. 3, indicated that, compared to marker enzymes of the cytosol (formaldehyde dehydrogenase) and mitochondria (citrate synthase), the behaviour of DHAS is typical for that of a peroxisomal enzyme. The decrease in activity of formaldehyde dehydrogenase was explained by dilution of enzyme protein over newly formed cells following inhibition of its synthesis after glucose addition.

Electron microscopy and immunocytochemistry

Cells of *H. polymorpha* from cultures in the exponential phase of growth on methanol contained several peroxisomes which had large crystalline cores (Fig. 4). These organelles were enriched in the $30,000 \times g$ pellet, obtained after differential centrifugation of cell homogenates (for details see Materials and methods). In addition this fraction contained vacuoles, membrane vesicles and mitochondria (Fig. 5). The band of protein observed after sucrose gradient centrifugation of the $30,000 \times g$ fraction at approximately 52% sucrose, which was characterized by high activities of alcohol oxidase, catalase and DHAS, contained highly purified microbodies (Fig. 6, 7). Judged by their morphology the majority of the isolated organelles were virtually intact (compare Fig. 4 and Fig. 6). A small number appeared to be more or less swollen and in a number of cases part of the soluble proteins present in the organelles probably had leaked. However, the crystalline cores were generally unaffected (Fig. 7). After immunogold labelling carried out on ultrathin sections of glutaraldehyde-fixed cells embedded in Lowicryl K4M with antibodies against DHAS, gold

Fig. 3A–C. Growth and enzyme activities in cell-free extracts of *H. polymorpha* after addition of 1.0% (w/v) glucose to cultures in the exponential phase of growth on methanol. **A** Growth ($\odot$), expressed as optical density at 663 nm. **B** Alcohol oxidase ($\square$), catalase ($\circ$) and DHAS ($\blacksquare$) activity. **C** Formaldehyde dehydrogenase ($\blacktriangledown$) and citrate synthase ($\square$) activity. Alcohol oxidase is expressed as $\mu\text{mol O}_2 \text{ consumed} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, catalase as $\Delta E_{240} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, DHAS as $\mu\text{mol formaldehyde consumed} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, formaldehyde dehydrogenase as $\mu\text{mol NADH formed} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, and citrate synthase as $\mu\text{mol citrate formed} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

particles were almost exclusively confined to the peroxisomes and were randomly distributed over the peroxisomal matrix (Fig. 8). Similar labelling patterns were obtained when antibodies against alcohol oxidase were used. After double immunogold labelling of both DHAS and alcohol oxidase, using antibodies against these enzymes which were conjugated with protein A and gold particles of different diameters (3 nm and 11 nm, respectively) almost all gold particles were present within the peroxisomal boundary (Fig. 9).

Discussion

The results presented in this study clearly indicate that in methanol-grown *Hansenula polymorpha* dihydroxyacetone synthase (DHAS) is compartmentalized in peroxisomes. In our immunocytochemical experiments the gold particles were present in all the peroxisomal profiles, were detected in all thin sections examined and were randomly distributed over the peroxisomal matrix. Therefore, all the organelles present in the cells contain this enzyme. Our results also indicate that DHAS and alcohol oxidase are the two major proteins of these organelles. Previously we have shown (see Veenhuis et al. 1983) that peroxisomes of methanol-grown

H. polymorpha may possess a partial or completely crystalline matrix composed of alcohol oxidase protein molecules and that the size of the crystal is determined by growth conditions. It is very unlikely that DHAS forms a structural part of the peroxisomal crystalloid. Instead it is most probably present in a soluble, mobile form as has been suggested for catalase (Veenhuis et al. 1981).

Our present results are in agreement with the results reported by Goodman et al. (1984) with methanol-grown *Candida boidinii*. As in *H. polymorpha* (Fig. 2) peroxisomes prepared from *C. boidinii* contained two major proteins, one of which was identified as alcohol oxidase. The other — unidentified — protein had a similar subunit molecular weight (79 kDa) as that of the DHAS of *H. polymorpha* reported in the present study. Essentially similar results were obtained during other investigations (Roa and Blobel 1983; Roggenkamp et al. 1984). For this reason it appeared very likely that also in *C. boidinii* — as probably in other methylotrophic yeasts — DHAS is a peroxisomal matrix enzyme. In fact Goodman (1985) very recently provided evidence that in *C. boidinii* DHAS occurs in peroxisomes. Therefore the observations of Jenkins et al. (1985), who on the basis of subcellular fractionation experiments suggested that DHAS in *C. boidinii* is localized in mitochondria, probably must be explained by leakage of enzyme proteins from damaged organelles. This is certainly possible since the electron micrographs, of the different subcellular fractions accompanying this study do not show the crystalloids which are so characteristic for peroxisomes of methanol-grown yeasts.

The localization of the key enzyme of methanol assimilation in peroxisomes emphasizes the significance of these organelles in methanol metabolism. So far yeast microbodies were thought to be predominantly involved in catabolic processes (Veenhuis et al. 1983). However, the number of peroxisome-borne enzymes with a biosynthetic function (Zwart et al. 1983) is now gradually increasing. Recently it was observed that in addition to glutamate oxaloacetate aminotransferase, NAD-dependent glutamate dehydrogenase is present in peroxisomes of ethylamine-grown yeasts. Both enzymes are involved in the biosynthesis of aspartate by these organelles (Marten Veenhuis, unpublished).

The peroxisomal nature of DHAS may also resolve one of the major problems that was associated with its presumptive activity in vivo in the presence of excess glutathione. So far it was thought that the enzyme was cytoplasmic and thus it had to function under conditions where comparatively high concentrations of glutathione were present. In methylotrophic yeasts the dissimilation of formaldehyde occurs in the cytoplasm and is dependent on its condensation with reduced glutathione (GSH). The resulting hemimercaptal is in fact the substrate for formaldehyde dehydrogenase (van Dijken et al. 1976b; Veenhuis et al. 1983). In vitro experiments have shown that purified DHAS is strongly inhibited by GSH; when in the assay system the concentration of GSH exceeded that of formaldehyde by a factor of 2–3, DHAS activity was no longer detectable (Bystrykh et al. 1981). In vivo the ratio GSH/formaldehyde may far exceed this value. For instance, in methanol-grown *C. boidinii* the cytoplasmic concentration of GSH was at least 20 times that of formaldehyde (Ubiivovk et al. 1983). An explanation for the fact that DHAS can be active during growth on methanol can now be put forward on the basis of its compartmentalization (in

Figs. 4–9. Electron micrographs. *Abbreviations:* *m* mitochondrion, *v* vacuole, *p* peroxisome. The marker represents 0.5 μm .

Fig. 4. Survey of a protoplast prepared from a suspension of *H. polymorpha* cells taken from the exponential growth phase on methanol ($\text{OD}_{663} = 1.8$), showing the substructure of intact peroxisomes in these cells

Fig. 5. Thin section of the 30,000 $\times g$ pellet, obtained after differential centrifugation of cell homogenates of *H. polymorpha*. Apart from other cell constituents such as vacuoles, mitochondria and unidentified membrane vesicles many peroxisomal profiles are observed

Figs. 6, 7. Thin sections of the peroxisomal peak fraction (fraction 19) after sucrose gradient centrifugation. This fraction mainly contains peroxisomes (**Fig. 6**), the majority of which are intact (**Fig. 7**). However, some of the organelles appeared swollen and part of the soluble matrix enzymes had apparently leaked (**Fig. 6**; arrows)

Figs. 8, 9. Immunolabelling experiments. After immunolabelling of thin sections of Lowicryl embedded cells using antibodies against DHAS coupled with protein A-gold, specific labelling is only present in the peroxisomal matrix (**Fig. 8**). Similar results are obtained after double immunolabelling of DHAS and alcohol oxidase by the same method (**Fig. 9**; alcohol oxidase — 11 nm gold particles; DHAS — 3 nm gold particles [arrow])

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