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Methanol metabolism in a peroxisome-deficient mutant of *Hansenula polymorpha*: a physiological study

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Abstract. We have studied methanol-utilization in a peroxisome-deficient (PER) mutant of *Hansenula polymorpha*. In spite of the fact that in carbon-limited chemostat cultures under induced conditions the enzymes involved in methanol metabolism were present at wild-type (WT) levels, this mutant is unable to grow on methanol as a sole carbon and energy source. Addition of methanol to glucose-limited ($S_R = 12.5$ mM) chemostat cultures of the PER mutant only resulted in an increase in yield when small amounts were used (up to 22.5 mM). At increasing amounts however, a gradual decrease in cell density was observed which, at 80 mM methanol in the feed, had dropped below the original value of the glucose-limited culture. This reduction in yield was not observed when increasing amounts of formate instead of methanol were used as supplements for the glucose-limited mutant culture and also not in WT cells, used as control in these experiments. The effect of addition of methanol to a glucose-limited PER culture was also studied in the transient state during adaptation of the cells to methanol. The enzyme patterns obtained suggested that the ultimate decrease in yield observed at enhanced methanol concentrations was due to an inefficient methanol-metabolism as a consequence of the absence of peroxisomes. The absence of intact peroxisomes results in two major problems namely i) in H_2O_2 -metabolism, which most probably is no longer mediated by catalase and ii) the inability of the cell to control the fluxes of formaldehyde, generated from methanol. The energetic consequences of this metabolism, compared to the WT situation with intact peroxisomes, are discussed.

Key words: *Hansenula polymorpha* — Peroxisomes — Peroxisome function — Peroxisome-deficient mutant — Methanol metabolism — Continuous cultures — Mixed-substrates

Offprint requests to: I. J. van der Klei

Abbreviations: AO, alcohol oxidase; DHAS, dihydroxyacetone synthase; WT, wild-type; PER, peroxisome-deficient; GSH, reduced glutathione; GSSG, glutathione disulphide

Microbodies (peroxisomes, glyoxysomes) represent a class of subcellular organelles, which are present in almost all eukaryotic cells (Fahimi and Sies 1987). The significance of these organelles in cellular metabolism was not considered very important during the first years following their discovery (Rodin 1954). However, later studies stressed the importance of the organelles and revealed that they may be involved in a variety of metabolic functions (Fahimi and Sies 1987; Borst 1989). The vital importance of microbodies in cellular metabolism is now fully acknowledged; the absence of the organelles in mammalian cells invariably leads to severe abnormalities and often is lethal [e.g. the hereditary disease Zellweger syndrome and related disorders (Goldfischer et al. 1973; Wanders et al. 1988)]. However, the advantage for an organism to compartmentalize certain metabolic functions in microbodies is still a matter of speculation. Yeasts turned out to be appropriate model organisms to study microbody biogenesis and function, because their proliferation and enzyme composition can readily be manipulated by varying growth substrates (Zwart 1983; Fukui and Tanaka 1979; Veenhuis and Harder 1988, 1989). For instance, in *Hansenula polymorpha*, the initial metabolism of methanol and special nitrogen sources such as alkylated amines or D-amino acids is catalyzed by microbody-based enzymes. When these compounds are utilized microbody proliferation is invariably observed. One possible approach to elucidate microbody function involves physiological studies using mutants, defective in the assembly of these organelles. Such mutants have recently been isolated from the yeasts *Saccharomyces cerevisiae* (PAS-mutants, Erdmann et al. 1989) and *H. polymorpha* (PER-mutants, Cregg et al. 1990). The isolation procedure for these mutants was based on the same strategy, namely electron microscopical screening of mutants which were defective in the utilization of carbon sources, which in wild type (WT) cells are metabolized by microbody-born enzymes. Genetic analysis of the PER mutants confirmed that the absence of intact peroxisomes and the impairment to

utilize methanol as a sole carbon and energy source can be due to a mutation in a single gene (Cregg et al. 1990).

Surprisingly, not all functions which are peroxisome-born in WT cells, are impaired in the PER-mutants. For instance, these cells are still able to grow in complex media in the presence of D-amino acids and alkylated amines, although the growth rate is slightly reduced compared to WT cells (Sulter et al. 1990). In fact, in the PER mutants all peroxisomal enzymes studied so far, including those involved in methanol metabolism, were found to be inducible and appeared to be present and active in the cytosol of these mutants (Sulter et al. 1990; van der Klei et al. 1991). High expression of alcohol oxidase (comparable to WT levels) was observed in glucose-limited chemostat cultures in the presence of choline as a sole nitrogen source (Zwart et al. 1983). In these cells alcohol oxidase was present in a large cytosolic crystalloid, which also harboured bulk of the dihydroxyacetone synthase protein (van der Klei et al. 1991). Since also low catalase activities were demonstrated inside these crystalloids, the major difference between intact organelles in WT cells and the crystalloids is the absence of the bounding peroxisomal membrane in the PER-mutant.

Now that conditions could be created in which the key enzymes in methanol-metabolism are associated in the mutant as in WT cells, the question arose whether such cells could be adapted to the utilization of methanol. This intriguing question was approached by a gradual replacement of glucose by methanol in chemostat cultures growing carbon- and energy-limited on glucose/choline. We also studied the effect of addition of increasing amounts of methanol to glucose-limited cultures of the PER-mutant. The results presented in this paper indicate that irrespective of the growth conditions the PER mutant of *H. polymorpha* cannot be adapted to utilize methanol as a sole source of carbon and energy. Depending on the amounts of methanol added, this compound can, however, be used as an additional energy source during glucose-limited growth. The impact of the absence of the peroxisome on the efficiency of methanol-metabolism in the PER-mutant, compared to the WT strain, is discussed.

Materials and methods

Micro-organisms and growth conditions

Wild-type (WT) *Hansenula polymorpha* CBS 4732 and the peroxisome-deficient (PER) mutant 125-2E were grown in continuous cultures at a dilution rate (D) of 0.1 h^{-1} , as described by van Dijken et al. (1976), using glucose as limiting carbon source (0.25% w/v) and ammonium sulphate (0.2% w/v) or choline (0.25% w/v) as the sole nitrogen source. Methanol or formate were added to the feed of such cultures, when they had reached the steady state, at concentrations ranging from 5–80 mM. Growth was monitored by measuring the optical density at 660 nm in a Vitatron colorimeter. Samples were taken from the transient state at different time points after addition of methanol or from steady-state cultures; the latter were assumed to be achieved after at least 5 volume displacements.

Preparation of crude extracts and enzyme assays

Crude extracts were prepared by shaking cell suspensions [approximately 50% (wet weight/w)] in 50 mM potassium phosphate buffer

pH 7.0 with acid washed glass beads (diameter 0.1 mm) on a Vortex shaker 6 times 1 min, with 1 min time intervals on ice. Glass beads and cell debris were removed by centrifugation in an Eppendorf microfuge, maximal speed for 10 min at room temperature. Alcohol oxidase (AO) was assayed in crude extracts as described by Verduyn et al. (1984); in addition methanol dependent oxygen consumption by whole cells ($Q_{O_2}^{\max}$) was determined using a biological oxygen monitor (BOM) as described by van Dijken et al. (1976). Catalase was assayed according to Lück (1963), formaldehyde dehydrogenase and formate dehydrogenase as described by van Dijken et al. (1976). The formaldehyde dehydrogenase assay was started by addition of crude extract instead of formaldehyde. Glutathion reductase was assayed as described by Goldberg and Spooner (1983). Formaldehyde reductase was measured by determining the decrease of absorbance at 340 nm in an assay mixture containing 20 mM formaldehyde, 0.2 mM NADH in 0.1 M potassium phosphate buffer pH 7.0. Dihydroxyacetone synthase was assayed in crude extracts prepared in 50 mM potassium phosphate buffer pH 6.8 containing 0.5 mM EDTA, 5 mM MgCl_2 and 0.5 mM DTT, which were dialyzed for 2 h against the same buffer prior to the assay. DHAS activity was determined using the discontinuous method with dinitrophenylhydrazine as described by Bystrykh et al. (1990).

All enzyme assays were performed at 37°C. Activities are expressed as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein, except for catalase which is expressed as $\Delta A_{240} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein. Oxygen consumption rates by whole cells are expressed as $\mu\text{mol O}_2 \text{ consumed} \cdot \text{min}^{-1} \cdot A_{660}^{-1}$.

Analytical methods

Residual substrate and metabolic intermediates in the culture fluid were determined after removal of intact cells by centrifugation in an Eppendorf microfuge, maximal speed, 5 min. Methanol was determined by the enzymatic method described by Verduyn et al. (1984) or by gas chromatography (Laanbroek et al. 1982), formaldehyde according to the method of Nash (1953), formate as described by Lang and Lang (1972) and glucose by the glucose oxidase/peroxidase method (Boehringer, Mannheim, FRG). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard.

Electron microscopy

Spheroplasts, prepared according to Douma et al. (1985), were fixed in 6% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 60 min at 0°C, followed by postfixation in a mixture of 0.5% (w/v) OsO_4 and 2.5% (w/v) $\text{K}_2\text{Cr}_2\text{O}_7$ in the cacodylate buffer for 90 min at 0°C. After dehydration in a graded ethanol series the samples were embedded in Epon 812; ultrathin sections were cut with a diamond knife and examined in a Philips EM 300 or CM10.

Results

Cell morphology

Cells of the peroxisome-deficient (PER) mutant of *H. polymorpha* contain large cytosolic alcohol oxidase crystalloids during growth in a carbon-limited chemostat on glucose/choline (van der Klei et al. 1991). Such structures were absent in parallel cultures growing on glucose/ammonium sulphate, but rapidly appeared when methanol was added to the feed of such cultures. Already at low amounts of methanol (10 mM), crystalloids were observed in almost all individual cells within a period of

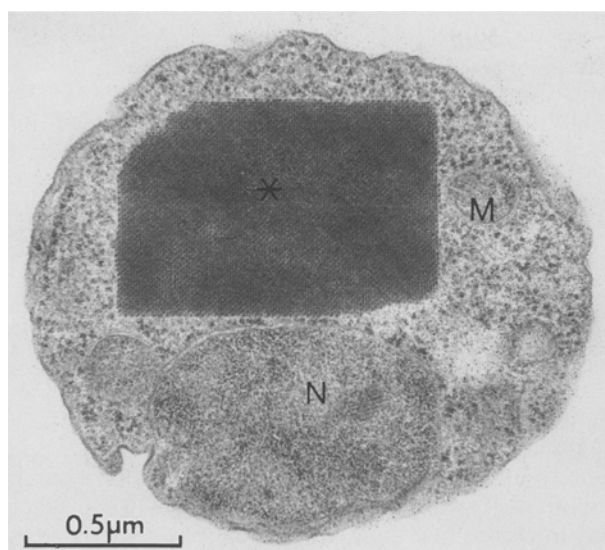


Fig. 1. PER cells grown in glucose-limited continuous culture, 10 h after addition of 10 mM methanol to the feed. The picture shows the presence of a large intracellular inclusion consisting of crystallized alcohol oxidase (AO) protein (glutaraldehyde/OsO₄-fixed protoplast; *, cytosolic AO crystalloid, *M*, mitochondrion, *N*, nucleus)

10 h (Fig. 1). Immunocytochemically these structures contained alcohol oxidase (AO) and dihydroxyacetone synthase (DHAS), identical to those described for glucose/choline-grown cells (van der Klei et al. 1991; results not shown).

Growth and enzyme patterns in glucose-limited chemostat cultures

Steady-state experiments. In an initial series of experiments, we investigated whether cells of the PER mutant of *H. polymorpha*, precultured on glucose/choline so that they contained large cytosolic AO crystalloids (van der Klei et al. 1991), could be adapted to growth on methanol alone. No growth was observed when such cells were transferred to batch cultures containing methanol as the sole carbon source. In glucose-limited continuous cultures only the addition of small amounts of methanol [less than 0.1% (v/v) final concentration] to the feed led to a slight increase in cell densities. A drastic inactivation of AO was observed upon further addition of methanol (data not shown). All attempts to gradually replace glucose by methanol, by a stepwise dilution of the feed containing a glucose/methanol mixture with medium containing methanol alone, failed. Under these conditions the densities of the cultures decreased concomitantly with the decrease in glucose concentrations in the feed and finally the cultures were washed out when glucose concentrations reached zero. One possible explanation for these results could be related to the original high AO activities, present in the culture prior to the addition of methanol: inactivation of this enzyme under methanol excess conditions is a well-known phenomenon (Bruinenberg et al. 1982).

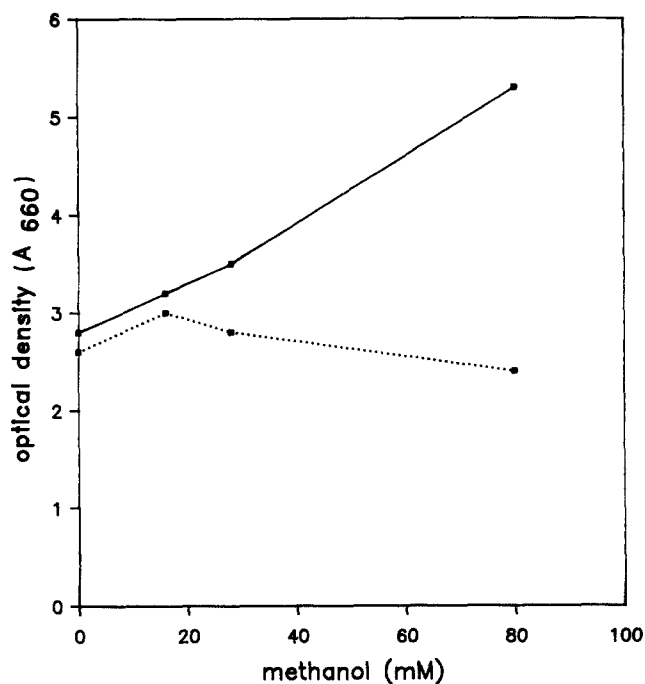


Fig. 2. Effect of increasing concentrations of methanol (0–80 mM) in the feed of glucose-limited continuous cultures ($S_R = 12.5$ mM) ($D = 0.1$ h⁻¹) of *Hansenula polymorpha* wild type (WT) and peroxisome-deficient (PER) mutant cells on the cell yield in steady state cultures (■—■ WT; ■---■ PER)

For this reason we studied the effect of addition of different amounts of methanol to the feed of a continuous culture ($D = 0.1$ h⁻¹) of the PER mutant on glucose/ammonium sulphate. Under these conditions the expression of AO in wild-type (WT) cells is relatively low (Egli et al. 1980). The results are summarized in Fig. 2. The WT strain, grown as control, showed the expected linear increase in biomass with increasing amounts of methanol (Egli et al. 1982). However, the PER mutant showed a different response. Only at small amounts of methanol an increase in cell density was observed; this decreased gradually upon increasing amounts of methanol and, at 80 mM methanol, resulted in cell densities which were below the original density of the culture prior to the addition of methanol (Fig. 2). In both cultures the residual glucose and methanol concentrations were determined. Glucose invariably was undetectable under all conditions; methanol was only detected in the culture fluid of the PER mutant at enhanced methanol concentrations; in cultures supplemented with 80 mM methanol in the feed approximately 20 mM of this compound was present in the culture fluid at steady-state conditions.

In order to clarify whether the observed decrease in biomass of the PER strain was specific for methanol (e.g. a toxic effect caused by H₂O₂-accumulation), we studied the addition of formate, an intermediate of methanol dissimilation, on the yield of the PER-culture. As shown before (Babel et al. 1983) formate can be used as an additional energy source by glucose-limited cultures of WT cells. A similar response was found for the PER strain. Addition of formate to the feed of a glucose-limited continuous culture of the PER mutant was paral-

Table 1. Alcohol oxidase activities in steady-state cells from glucose-limited continuous cultures before and after addition of different methanol concentrations

Methanol concentration (mM)	Wild-type		PER mutant	
	Crude extract	$Q_{O_2}^{\max}$	Crude extract	$Q_{O_2}^{\max}$
0	0.6	—	0.5	—
22.5	5.0	—	4.6	—
80	7.7	0.15	5.0	0.13

AO activities, determined in crude extracts, are expressed as $U \cdot mg^{-1}$ protein. Methanol-dependent oxygen consumption rates were measured using whole cells and are expressed as $\mu mol \cdot O_2$ consumed $\cdot min^{-1} \cdot A_{660}^{-1}$ (— = not determined)

led with a gradual increase in cell density, a pattern comparable to WT cells. The initial A_{660} of the PER culture, which amounted to 2.9 in the absence of formate increased to 3.4 at 40 mM formate and 3.6 at 80 mM formate. One possible explanation for the presence of residual methanol concentrations in the culture fluid of the PER culture, when grown in a glucose-limited chemostat in the presence of 80 mM methanol, may involve a selective inactivation of AO under these conditions. As shown before, the activity of the enzyme is sensitive to H_2O_2 (Kato et al. 1976), but is also inhibited by excess methanol (Bruinenberg et al. 1982). For this reason specific AO activities were determined in cells from the various growth conditions. The results, summarized in Table 1, show that in both the WT and the PER mutant strain AO synthesis was derepressed in glucose-limited cultures. Addition of methanol to the feed caused an additional increase in AO activity, which was dependent on the amount of methanol added, both in WT and PER cultures. We furthermore determined the methanol-dependent oxygen consumption rates ($Q_{O_2}^{\max}$) since AO may (partly) be inactive under in vivo conditions (Veenhuis and Harder 1989) and the activities found be due to reactivation of the enzyme upon the preparation of crude extracts. However, both WT and PER cells displayed comparable oxygen consumption rates upon addition of methanol to intact cell suspensions (Table 1) indicating that also in vivo high activities of AO were present in cells of both strains.

Transient state experiments

The results, obtained with the chemostat cultures did not permit an answer to two major questions, namely why i) methanol was not fully consumed in the PER cultures which displayed high AO activities and ii) increasing amounts of methanol, supplemented to the PER cultured during growth in a glucose-limited chemostat, led to a reduction in biomass of the culture. To clarify these points, we decided to study the response of both the WT and the PER cultures in the transient state during adaptation of cells to methanol. For this purpose methanol was added at a final concentration of 75 mM to

the feed of glucose-limited steady-state cultures ($D = 0.1 h^{-1}$). The effect on the cell densities of both cultures is shown in Fig. 3A. The WT strain showed the expected response: the optical density (A_{660}) of the culture increased gradually during the first 32 h after the addition of methanol from 3.4 to 6.4 and remained constant thereafter. In the PER culture a minor increase in cell density was observed during the initial 16 h (from 2.9 to 3.4), which was followed by a gradual decrease to 3.1 40 h after the start of methanol addition. A “steady-state” was reached after 50 h. However, in the PER culture a further decrease in biomass was observed after prolonged cultivation (over 100 h) to a value of 2.7, which is below the initial value of the glucose-limited culture prior to methanol addition.

The residual amounts of methanol and intermediary metabolites (formaldehyde and formate) were determined in the culture fluid. In WT cultures low residual amounts of methanol were only observed during the first 4 h after addition (Fig. 3B); the highest value (3 mM) was detected after 2 h. However, in the PER culture methanol was present in all samples, although at low concentrations (Fig. 3B); as in WT cells, the highest concentration (5.8 mM) was observed during the initial hours after addition of methanol to the feed. The subsequent gradual decrease continued after the culture had reached a “steady-state” (after 50 h) to finally 1 mM (after 75 h). Formaldehyde, the first product of methanol oxidation was also detected in all samples of the PER culture. This showed a fluctuating pattern in the initial period and remained present at low levels thereafter (Fig. 3C). In WT cultures formaldehyde, like methanol, was only detectable during the first 4 h after methanol addition (Fig. 3C). The patterns of the residual amounts of formate, the second intermediate of the dissimilation pathway, are depicted in Fig. 3D. After the shift the formate concentrations initially fluctuated in both cultures during the adaptation of cells to the new environment; however, at steady-state conditions very low amounts were found (Fig. 3D).

The enzyme patterns of the different enzymes involved in methanol metabolism are depicted in Fig. 4. As expected, AO activity, which was present at low levels in both the PER and WT strain during glucose-limited growth, gradually increased until levels were reached of approximately $5.5 U \cdot mg^{-1}$ protein after 40 h (Fig. 4A). For catalase, formaldehyde and formate dehydrogenase (Fig. 4B–D) significant differences were observed during the initial adaptation period of both strains; especially catalase activity drastically increased during the initial 6 h following addition of methanol to the PER-cultures. However, all three enzymes displayed comparable activities when the cultures had reached steady-state conditions (Fig. 4B–D).

Dihydroxyacetone synthase (DHAS), the key enzyme of methanol assimilation, was absent in the glucose-grown cells, but was rapidly induced by methanol and reached values of 0.27 and 0.21 $U \cdot mg^{-1}$ protein in the WT and PER strain, respectively, after 32 h.

Two other enzymes, which may be involved in methylotrophic growth of *H. polymorpha* were measured,

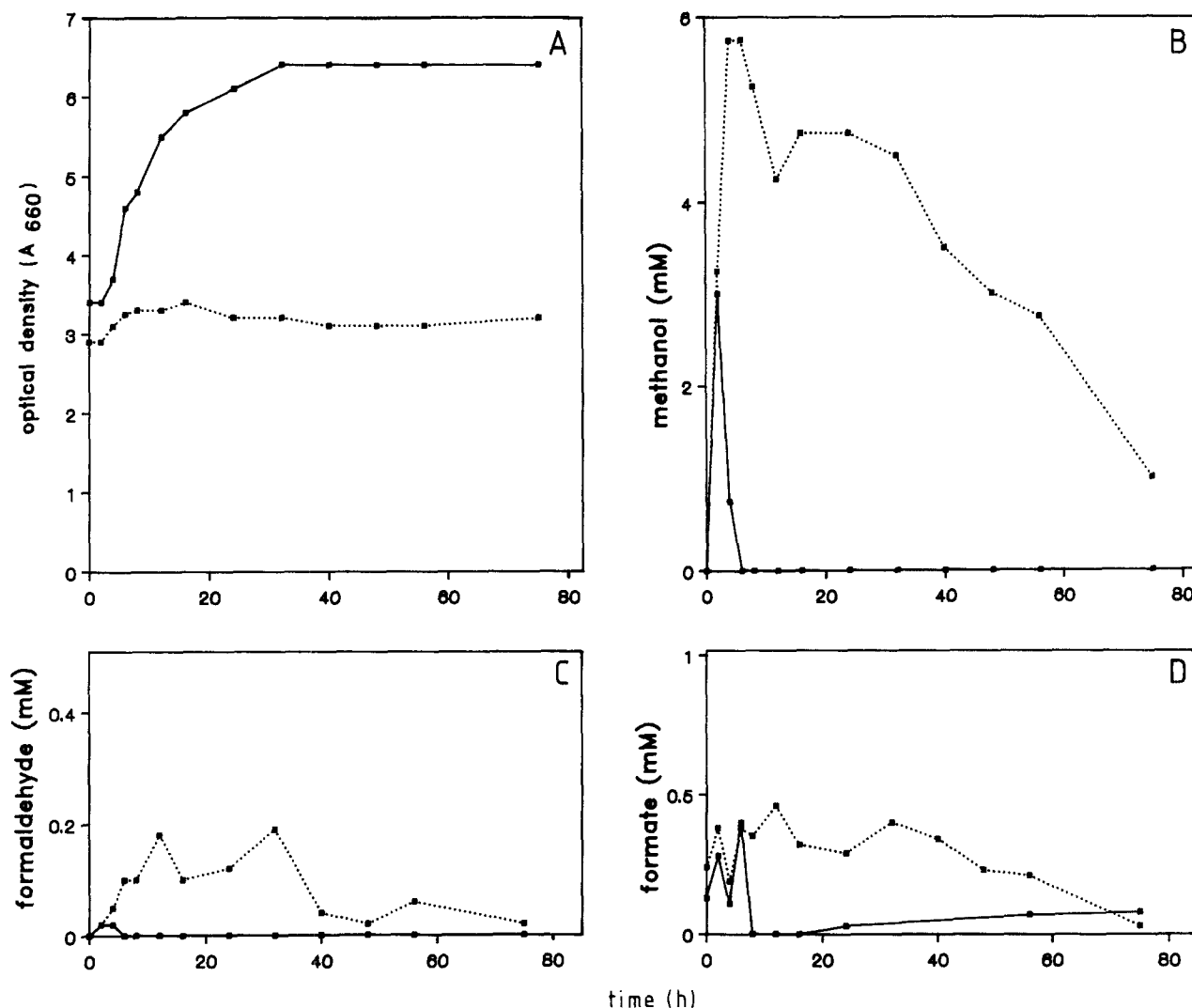


Fig. 3. Cell yields (A) and concentrations of methanol (B), formaldehyde (C) and formate (D) in the transient state of glucose-limited continuous cultures ($D = 0.1 \text{ h}^{-1}$) following addition of methanol (75 mM) to the feed ($t = 0 \text{ h}$) in *H. polymorpha* WT and PER cultures (■—■ WT; ■---■ PER)

namely formaldehyde reductase and glutathione reductase. Formaldehyde reductase reduces formaldehyde to methanol and was suggested to play an important role in the control of cytosolic formaldehyde concentrations under conditions when these are significantly increased compared to the levels normally found (Sibirny et al. 1988). In WT cells the activity of formaldehyde reductase strongly fluctuated (between values of 1.5 and $6 \text{ U} \cdot \text{mg}^{-1}$ protein) in the initial period after the addition of methanol, but was restored to the level, originally present in the glucose-grown cells, during steady state conditions. In contrast, in the PER cultures the activity rapidly dropped and remained at very low levels thereafter (Fig. 5A). Consequently, formaldehyde reductase does probably not play a significant role in the regulation of enhanced formaldehyde concentrations in the PER mutant. Glutathione reductase, which catalyzes the NADPH-dependent reduction of glutathione disulphide (GSSG) into glutathione (GSH), initially was strongly induced by methanol in both cultures (Fig. 5B), but subsequently

decreased to constant levels. However, under “steady-state” conditions the activities of this enzyme, detected in PER cells, remained approximately 3-fold higher than those in WT cells.

Discussion

In this study we have shown that a peroxisome-deficient (PER) mutant of *H. polymorpha* can not be adapted to utilize methanol as a sole carbon and energy source, despite the fact that all peroxisomal enzymes involved in methanol-metabolism (namely AO, DHAS and catalase) are active and present at levels comparable to WT cells. A possible explanation of these results is based on the view that in WT cells peroxisomes are important for an appropriate partitioning of formaldehyde fluxes over dissimilatory and assimilatory pathways (Harder et al. 1987). In peroxisome-deficient cells formaldehyde can not be balanced by this mechanism, because formalde-

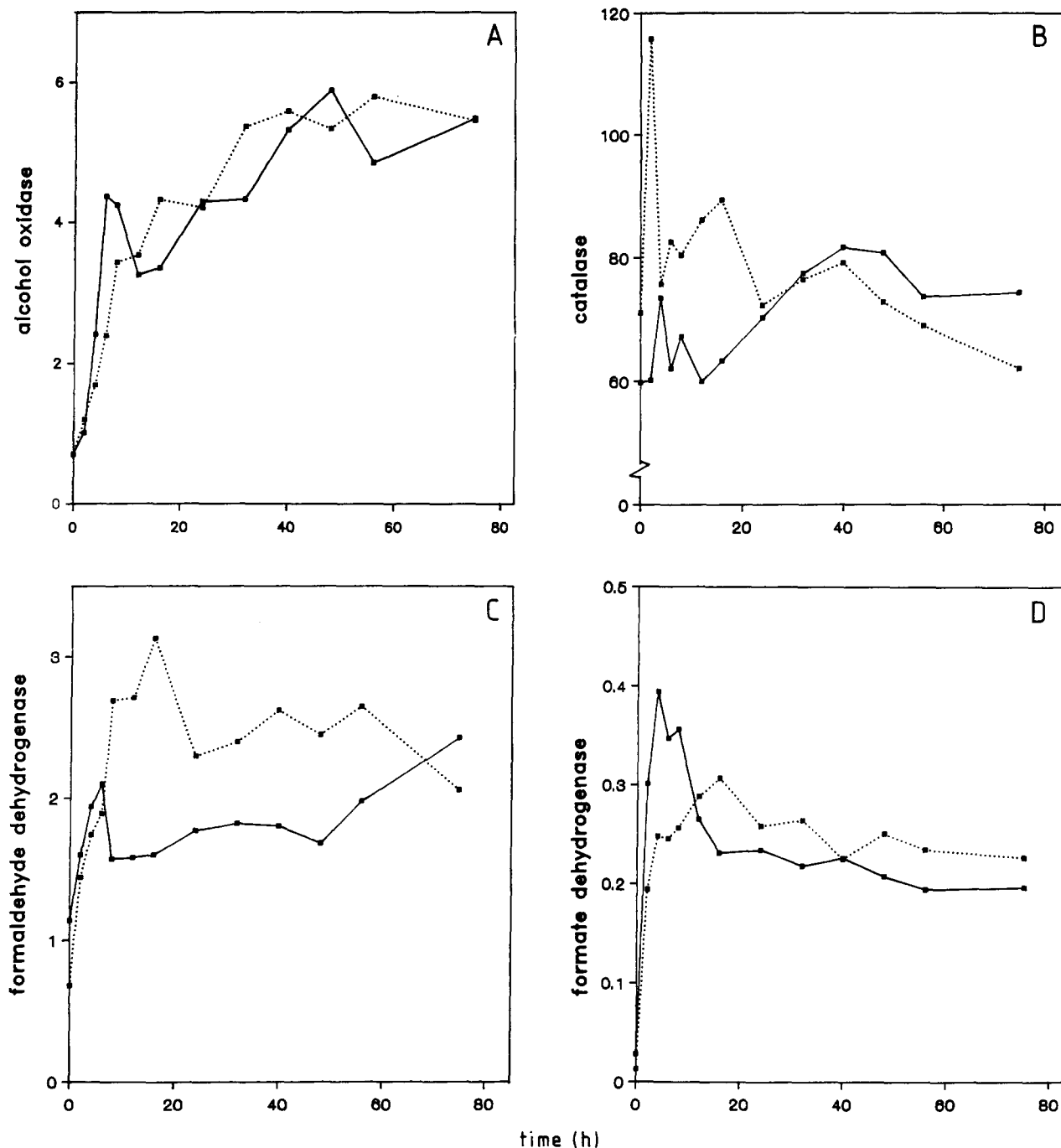


Fig. 4. Enzyme patterns of alcohol oxidase (A), catalase (B), formaldehyde dehydrogenase (C) and formate dehydrogenase (D) in the transient state of glucose-limited WT and PER cultures as described in Fig. 3. Activities are expressed as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein except for catalase which is expressed as $\Delta A_{240} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein (■—■ WT; ■---■ PER)

hyde, now generated in the cytosol, will rapidly react with glutathione (GSH) into S-hydroxymethyl-glutathione ($\text{GS-CH}_2\text{OH}$), which is the actual substrate for formaldehyde dehydrogenase (Schütte et al. 1976; Allais et al. 1983). However, in this form it is no longer accessible for assimilation by DHAS (Bystrykh et al. 1990). Consequently, little if any free formaldehyde will be available for assimilation, which may explain why methanol can not be used as a sole carbon source by PER mutants. On the other hand, at first sight there is no plausible

explanation as to why methanol can not be used effectively as an additional energy source by the PER-mutants. At the enzyme levels, no repression/inactivation of methanol dissimilatory enzymes was observed at the methanol concentrations used in these experiments. Thus, formaldehyde essentially should be available for dissimilatory purposes. However, this was only observed at low methanol supply rates. The decrease in cell densities observed at enhanced methanol availability indicates that its metabolism now may require energy and/or causes

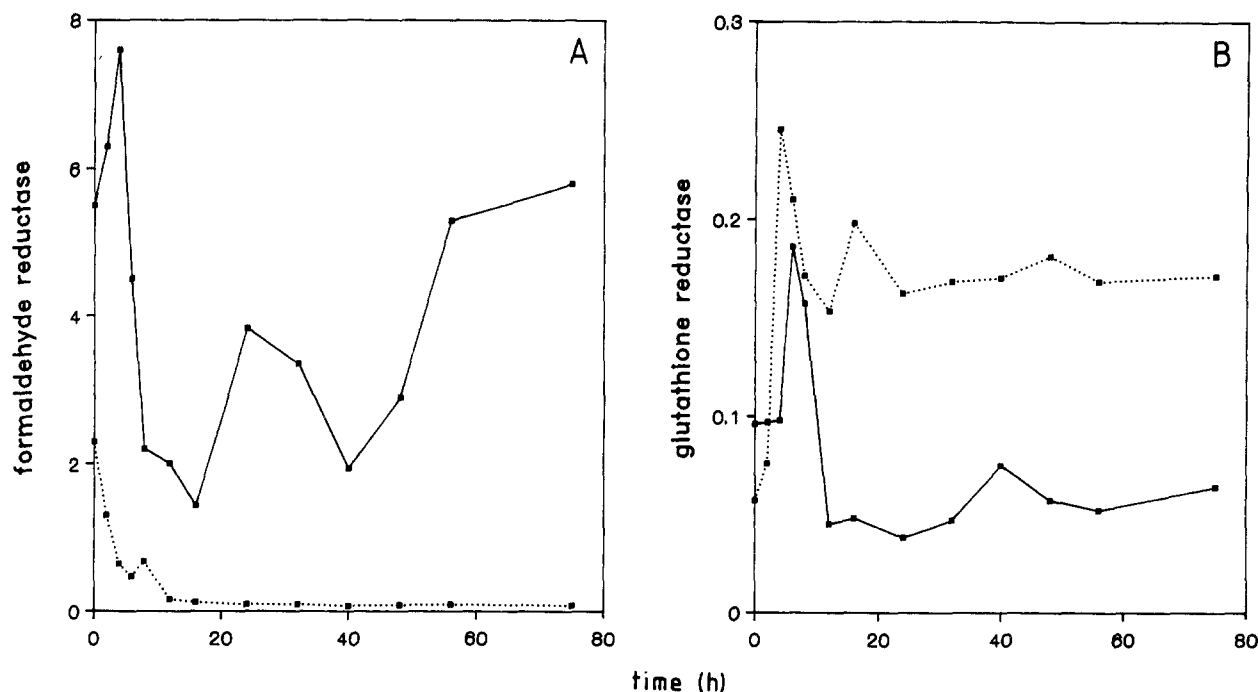


Fig. 5. Enzyme patterns of formaldehyde reductase (A) and glutathione reductase (B) in the transient state as described in Fig. 3. Activities are expressed as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{protein}$ (●—● WT; ●---● PER)

toxic defects. The observation that such inhibitory effects did not occur when, instead of methanol, formate was used as an additional energy source led us to the conclusion that they must be related to the metabolism of either one or both of the intermediates generated in the first step of methanol oxidation, namely formaldehyde and/or hydrogen peroxide.

In fact, a change in H_2O_2 -metabolism may provide a fundamental clue to the observed inefficiency of methanol dissimilation in the PER mutant. In WT cells, catalase is involved in the decomposition of H_2O_2 at the site of its production from methanol, namely inside peroxisomes. In the PER mutants, however, catalase is predominantly present in soluble form all over the cytosol; as a consequence the significance of the enzyme in H_2O_2 -decomposition may be of minor importance due to the relatively poor affinity of catalase for this substrate compared to other H_2O_2 -metabolizing systems as for instance cytochrome C peroxidase (CCP) or glutathion peroxidase. This assumption is not only hypothetical as is evident from the work of Verduyn et al. (1988), who showed that addition of H_2O_2 to a glucose-limited chemostat culture of WT *H. polymorpha* led to a decrease in yield.

Glutathione peroxidase was never detected in *H. polymorpha* (Verduyn et al. 1988; L. Bystrykh, unpublished results); however, GSH may also be oxidized non-enzymatically by the H_2O_2 generated in the cytosol of PER-mutants (Meister and Anderson 1983; Ross et al. 1985). Especially, at enhanced rates of methanol oxidation, chemical oxidation of GSH by H_2O_2 may significantly reduce the amount of free GSH available for condensation with formaldehyde generated at the same time. Limiting pools of free GSH would in turn lead to an

inefficient metabolism of the formaldehyde available under these conditions. Enhanced oxidation of GSH, which is also suggested by the high activities of glutathione reductase in the PER cells, may in fact explain our observations made on methanol utilization by these cells.

Although it is hard to explain as to why methanol is not fully consumed, despite the presence of high AO activities, this may partly be due to enhanced intracellular levels of free formaldehyde. In aqueous solutions free formaldehyde is not stable, but converted into its hydrated form, methylene glycol (99.9% is present in this form in aqueous solutions; Bieber and Trümpler 1947). Methylene glycol is not a substrate for either formaldehyde dehydrogenase or DHAS, but may be oxidized by AO into formate (Sahm 1975; van Dijken 1976). Therefore, accumulation of intracellular pools of methylene glycol may result in competition for the active sites of AO and thus, at least partly, explain why not all methanol is oxidized by the PER cells (Kato et al. 1976). However, as indicated in Fig. 3B, methanol concentrations are still decreasing at 75 h after addition of methanol. Consequently, it is likely that after prolonged cultivation times all methanol will be consumed. At low methanol concentrations in the feed no residual substrate was detected in the cultures; under these conditions the amount of GSH (normally present in excess) will not be limiting due to the relatively small amounts of H_2O_2 produced. Therefore, as long as GSH is not limiting, the dissimilation pathway will not be hampered. The net energy yield, namely 1 NADH/molecule methanol oxidized (2 NADH from oxidation of methanol into CO_2 minus the reduction equivalent required for H_2O_2 -decomposition via CCP (1 NADH) or oxidation/reduction of glutathione (1

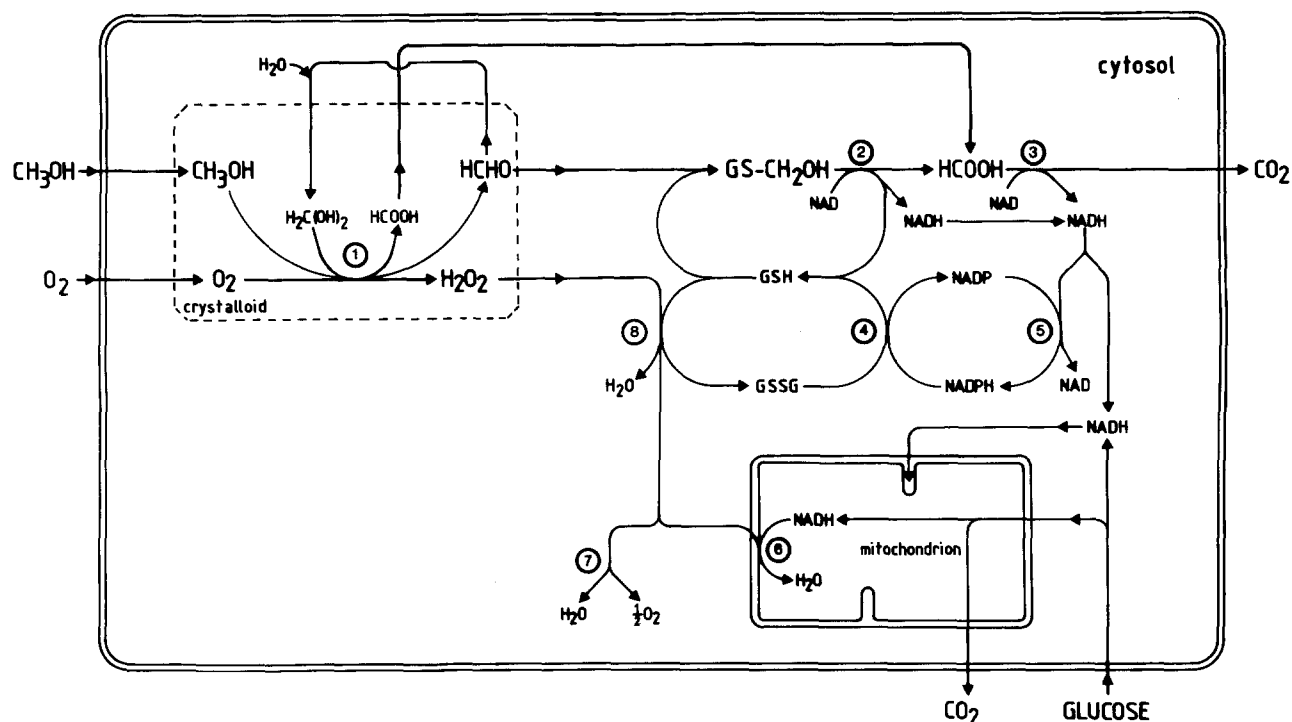


Fig. 6. Hypothetic scheme of methanol metabolism in the PER mutant during growth of cells in glucose-limited continuous cultures, supplemented with methanol as second substrate in the feed. 1. alcohol oxidase (catalyzes both the oxidation of methanol and methylene glycol, the hydrated form of formaldehyde), 2. formaldehyde dehydrogenase, 3. formate dehydrogenase, 4. glutathione

reductase, 5. NADH/NADPH transhydrogenase, 6. cytochrome C peroxidase, 7. catalase, 8. chemical oxidation of GSH by H_2O_2 . Enzymes which are thought to be of minor significance in methanol metabolism in the PER mutant (dihydroxyacetone synthase, formaldehyde reductase) are not included

NADPH) explains the increase in cell densities observed under these conditions. However, under GSH-limiting conditions (at enhanced methanol supply rates) the net energy yield may be negative. Oxidation of methanol to formaldehyde, which is subsequently hydrated and oxidized by AO to formate, results in the generation of 2 H_2O_2 (decomposed at the expense of two reduction equivalents), whereas only one NADH is formed from the oxidation of formate into CO_2 . Therefore, the final balance between the normal dissimilation pathway and the pathway via methylene glycol may determine a net energy yield, which can be negative at enhanced methanol supply rates. A schematic representation of the methanol metabolism in the PER-mutant is given in Fig. 6.

In summary, our results suggest that the main functions of peroxisomes in methanol metabolism are closely related and include i) decomposition (by catalase) of H_2O_2 at the site where it is produced, thus preventing decomposition of hydrogen peroxide by energy consuming processes (compare Fig. 6), thus enabling ii) proper partitioning of formaldehyde fluxed over the dissimilatory and assimilatory pathways.

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References

- Allais JJ, Louktibi A, Baratti J (1983) Oxidation of methanol by the yeast, *Pichia pastoris*, purification and properties of the formaldehyde dehydrogenase. *Agric Biol Chem* 47: 1509–1516
- Babel W, Müller RH, Markuske KD (1983) Improvement of growth yield of yeasts on glucose to the maximum by using an additional energy source. *Arch Microbiol* 136: 203–298
- Bieber R, Trümpel G (1947) Angenäherte spektroskopische Bestimmung der Hydratationsgleichgewichtskonstanten wässriger Formaldehydlösungen. *Helv Chim Acta* 30: 1860–1865
- Borst P (1989) Peroxisome biogenesis revisited. *Biochim Biophys Acta* 1008: 1–13
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72: 248–254
- Bruinenberg PG, Veenhuis M, Dijken JP van, Duine JA, Harder W (1982) A quantitative analysis of selective inactivation of peroxisomal enzymes in the yeast *Hansenula polymorpha* by high-performace liquid chromatography. *FEMS Microbiol Lett* 15: 45–50
- Bystrykh LV, Koning W de, Harder W (1990) Dihydroxyacetone synthase from *Candida boidinii* KD 1. In: Lidstrom ME (ed) *Hydrocarbons and methylotrophy. Methods in enzymology*, vol. 188. Academic Press, Orlando, Florida, pp 435–445
- Cregg JM, Klei IJ van der, Sulter GJ, Veenhuis M, Harder W (1989) Peroxisome deficient mutants of *Hansenula polymorpha*. *Yeast* 6: 87–97
- Dijken JP van (1976) Oxidation of methanol by yeasts. PhD Thesis, University of Groningen, The Netherlands
- Dijken JP van, Otto R, Harder W (1976) Growth of *Hansenula polymorpha* in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. *Arch Microbiol* 111: 137–144
- Douma AC, Veenhuis M, Koning W de, Evers M, Harder W (1985) Dihydroxyacetone synthase is localized in the peroxisomal matrix of methanol-grown *Hansenula polymorpha*. *Arch Microbiol* 143: 237–243

- Egli Th, Dijken JP van, Veenhuis M, Harder W, Fiechter A (1980) Methanol metabolism in yeasts: regulation of the synthesis of catabolic enzymes. *Arch Microbiol* 124:115–121
- Egli Th, Kaeppli O, Fiechter A (1982) Regulatory flexibility of methylotrophic yeasts in chemostat cultures: simultaneous assimilation of glucose and methanol at a fixed dilution rate. *Arch Microbiol* 131:1–7
- Erdmann R, Veenhuis M, Mertens D, Kunau WH (1989) Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 86:5419–5423
- Fukui S, Tanaka A (1979) Peroxisomes of alkane- and methanol-grown yeasts. Metabolic functions and practical applications. *J Appl Biochem* 1:171–201
- Fahimi HD, Sies H (1987) Peroxisomes in biology and medicine. Springer, Berlin Heidelberg New York
- Goldberg DM, Spooner RJ (1983) Glutathione reductase. In: Bergmeyer HU (ed) *Methods of enzymatic analysis*, vol III. Verlag Chemie, Weinheim Deerfield Beach Basel, pp 258–265
- Goldfischer S, Moor CL, Johnson AB, Spiro AJ, Valsamis MP, Wisniewski HK, Ritch RH, Norton WT, Rapi I, Garner LM (1973) Peroxisomal and mitochondrial defects in cerebro-hepato-renal syndrome. *Science* 182:62–64
- Harder W, Trotsenko YA, Bystrykh LV, Egli T (1987) Metabolic regulation in methylotrophic yeasts. In: Verseveld HW van, Duine JA (eds) *Microbial growth on C₁ compounds*. Martinus Nijhoff, Dordrecht, The Netherlands, pp 139–149
- Kato N, Omori Y, Tani Y, Ogata K (1976) Alcohol oxidases of *Kloeckera* sp and *Hansenula polymorpha*. Catalytic properties and subunit structures. *Eur J Biochem* 64:341–350
- Klei IJ van der, Sulter GJ, Harder W, Veenhuis M (1991) Expression, assembly and crystallization of alcohol oxidase in a peroxisome-deficient mutant of *Hansenula polymorpha*; properties of the protein and architecture of the crystals. *Yeast* 7:15–24
- Laanbroek HJ, Abee T, Voogd JL (1982) Alcohol conversions by *Desulfobulbus propionicus* Lindhorst in the presence and absence of sulfate and hydrogen. *Arch Microbiol* 133:178–184
- Lang E, Lang H (1972) Spezifische Farbreaktion zum direkten Nachweis der Ameisensäure. *Fresenius' J Anal Chem* 260:8–10
- Lück H (1963) Catalase. In: Bergmeyer HU (ed) *Methods in enzymatic analysis*. Academic Press, New York, pp 885–894
- Meister A, Anderson ME (1983) Glutathione. *Ann Rev Biochem* 52:711–760
- Nash T (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* 55:416–421
- Rodin H (1954) Correlation of ultrastructural organization and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney. *Aktiebolaget Godvil, Stockholm*
- Ross D, Cotgreave I, Moldéus P (1985) The interaction of reduced glutathione with active oxygen species generated by xanthine-oxidase-catalyzed metabolism of xanthine. *Biochim Biophys Acta* 841:278–282
- Schütte H, Flossdorf J, Sahn H, Kula MR (1976) Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida boidinii*. *Eur J Biochem* 62:151–160
- Sahn H (1975) Oxidation of formaldehyde by alcohol oxidase of *Candida boidinii*. *Arch Microbiol* 105:179–181
- Sulter GJ, Klei IJ van der, Harder W, Veenhuis M (1990) Expression and assembly of amine oxidase and D-amino acid oxidase in the cytoplasm of peroxisome-deficient mutants of the yeast *Hansenula polymorpha* during growth on primary amines or D-alanine as the sole nitrogen source. *Yeast* 6:501–509
- Sibirny AA, Titorenko VI, Gonchar MV, Ubiyovok VM, Ksheminskaya GP, Vitvitskaya OO (1988) Genetic control of methanol utilization in yeasts. *J Basic Microbiol* 28:293–319
- Veenhuis M, Mateblowski M, Kunau WH, Harder W (1987) Proliferation of microbodies in *Saccharomyces cerevisiae*. *Yeast* 3:77–84
- Veenhuis M, Harder W (1988) Microbodies in yeasts: structure, function and biogenesis. *Microbiol Sci* 5:347–351
- Veenhuis M, Harder W (1989) Occurrence, proliferation and metabolic function of yeast microbodies. *Yeast* 5:517–524
- Verduyn C, Dijken JP van, Scheffers WA (1984) Colorimetric alcohol assays with alcohol oxidase. *J Microbiol Methods* 2:15–25
- Verduyn C, Giuseppin MLF, Scheffers WA, Dijken JP van (1988) Hydrogen peroxide metabolism in yeasts. *Appl Environ Microbiol* 54:2086–2090
- Wanders RJA, Roermund CWT van, Schutgens RBH, Barth PG, Heymans HSA, Bosch H van den, Tager JM (1990) The inborn errors of peroxisomal β -oxidation: a review. *J Inher Metab Disease* 13:4–36
- Zwart KB (1983) Metabolic significance of microbodies in the yeasts *Candida utilis* and *Hansenula polymorpha*. PhD Thesis, University of Groningen, The Netherlands
- Zwart KB, Veenhuis M, Harder W (1983) Significance of yeast peroxisomes in the metabolism of choline and ethanolamine. *Antonie van Leeuwenhoek* 49:369–385