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Proliferation and metabolic significance of peroxisomes in *Candida boidinii* during growth on D-alanine or oleic acid as the sole carbon source

G. J. Sulter[1], H. R. Waterham[1], J. M. Goodman[3], and M. Veenhuis[2]

[1] Department of Microbiology and [2] Laboratory for Electron Microscopy, Biological Center, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands
[3] Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235, USA

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Abstract. We have studied the induction of peroxisomes in the methylotrophic yeast *Candida boidinii* by D-alanine and oleic acid. The organism was able to utilize each of these compounds as the sole carbon source and grew with growth rates of $\mu = 0.20\ h^{-1}$ (on D-alanine) or $\mu = 0.43\ h^{-1}$ (on oleic acid). Growth was associated with the development of many peroxisomes in the cells. On D-alanine a cluster of tightly interwoven organelles was observed which made up 6.3% of the cytoplasmic volume and were characterized by the presence of D-amino acid oxidase and catalase. On oleic acid rounded to elongated peroxisomes were dominant which were scattered throughout the cytoplasm. These organelles contained increased levels of β-oxidation enzymes; their relative volume fraction amounted 12.8% of the cytoplasmic volume.

Key words: *Candida boidinii* – Yeast – Peroxisomes – β-Oxidation – D-Amino acid oxidase

In yeasts microbodies (peroxisomes, glyoxysomes) generally develop in response to environmental stimuli (Zwart 1983; Veenhuis and Harder 1987). Peroxisomes undergo in particular massive proliferation during growth of certain yeasts on methanol or fatty acids as the sole carbon source (Fukui and Tanaka 1979; Veenhuis et al. 1987; Veenhuis and Harder 1988). Under these conditions the organelles play an indispensable role in growth since they contain the key enzymes involved in the metabolism of these substrates (Douma et al. 1985; Kunau et al. 1987; Veenhuis and Harder 1989). This general property, namely the inducible nature of the organelles and the ease of manipulating their enzymic contents by changing growth conditions, renders yeast favorable organisms for studies on peroxisome biogenesis and/or function. However, compared to other yeast species, methylotrophic yeasts such as *Candida boidinii* suffer from the disadvantage that high numbers of peroxisomes only are induced by methanol; apart from this low numbers of small organelles can be induced by a few organic nitrogen sources, e.g. purines and primary alkylated amines (Veenhuis and Harder 1987; Veenhuis et al. 1986). For this reason we have sought other suitable growth substrates that can be utilized by *C. boidinii* as a sole carbon source, the metabolism of which is known be mediated by peroxisomal enzymes in other yeasts and therefore, may be expected to cause peroxisomal proliferation. *C. boidinii* was used in this study (instead of another well-studied methylotrophic yeast, *Hansenula polymorpha*) since peroxisomes are relatively easily isolated from this organism with high purity (Goodman et al. 1984). In this paper we provide evidence that both D-alanine and oleic acid are very suitable substrates for the induction of peroxisomes in this organism.

Offprint requests to: M. Veenhuis

Material and methods

Microorganism and growth conditions

Candida boidinii (ATCC 32195) was used in all experiments. The organism was grown in batch cultures at 30° C in the mineral medium described previously (van Dijken et al. 1976). As carbon sources 0.5% (w/v) glucose, 0.3% (w/v) propionate or 0.1% (v/v) oleic acid [in the presence of 0.02% (v/v) TWEEN 80] were used. In addition cultures were grown on 0.6% (w/v) D-alanine, 0.1% (w/v) uric acid or 0.3% (v/v) ethylamine as the combined carbon and nitrogen source.

Preparation of spheroplasts

Spheroplasts were prepared by treatment of suspensions of whole cells with Zymolyase (Douma et al. 1985).

Cell fractionation studies

Cells of *C. boidinii* were grown in batch cultures on D-alanine or oleic acid and harvested in the late exponential growth phase; peroxisomes were purified by differential and sucrose gradient centrifugation (Douma et al. 1985).

Table 1. Specific activities of different peroxisomal enzymes in cells of *Candida boidinii*, grown in batch cultures on different carbon sources

Enzyme	Specific activity on		
	Glucose/NH_4^+	Oleic acid/NH_4^+	D-Alanine
Catalase	5.5	109	139
D-Amino acid oxidase	5.2	2.3	228
Alcohol oxidase	0	0	0
Isocitrate lyase	9.8	320	66
Malate synthase	150	200	470
Bifunctional enzyme	420	1810	200
3-Oxoacyl CoA thiolase	70	160	102

Cells were harvested at $A_{663} = 2.2$ (glucose) or 1.2 (D-alanine and oleic acid). Catalase is expressed as $\Delta E240/min \cdot mg\ protein$, *other activities as* $U \cdot 10^{-3}$/mg protein

Analytical procedures

Cell free extracts were prepared as described by van Dijken et al (1976). All enzyme assays were performed at 30° C. Oxidase activities were assayed as described by Verduyn et al. (1984): prior to the assay of D-amino acid oxidase, the cell free extract was incubated with 5 µM FAD for 5 min at room temperature; D-amino acid oxidase was then determined using 50 mM D-alanine as the substrate. Catalase was measured as described by Lück (1963), cytochrome C oxidase as described by Douma et al. (1985) and isocitrate lyase and malate synthase as described by Dixon and Kornberg (1959). Bifunctional enzyme, comprising enoyl CoA hydratase and L-3-hydroxyacyl CoA dehydrogenase, and 3-oxoacyl-CoA thiolase were assayed as described by Kionka and Kunau (1985). Enzyme activities are expressed as $U \cdot 10^{-3}$/mg protein, except for catalase activity which is expressed as $\Delta E_{240}/min \cdot mg$ protein. Protein was determined by the method of Bradford (1976), using bovine serum albumin as a standard. Sucrose concentrations were determined by measuring the refractory index.

Electron microscopy and cytochemistry

Whole cells, protoplasts and purified peroxisomal fractions were fixed and prepared for electron microscopy as described before (Veenhuis et al. 1979). Cytochemical staining procedures for the detection of catalase (van Dijken et al. 1975) and D-amino acid oxidase activities (Veenhuis et al. 1976) were carried out as described.

Results

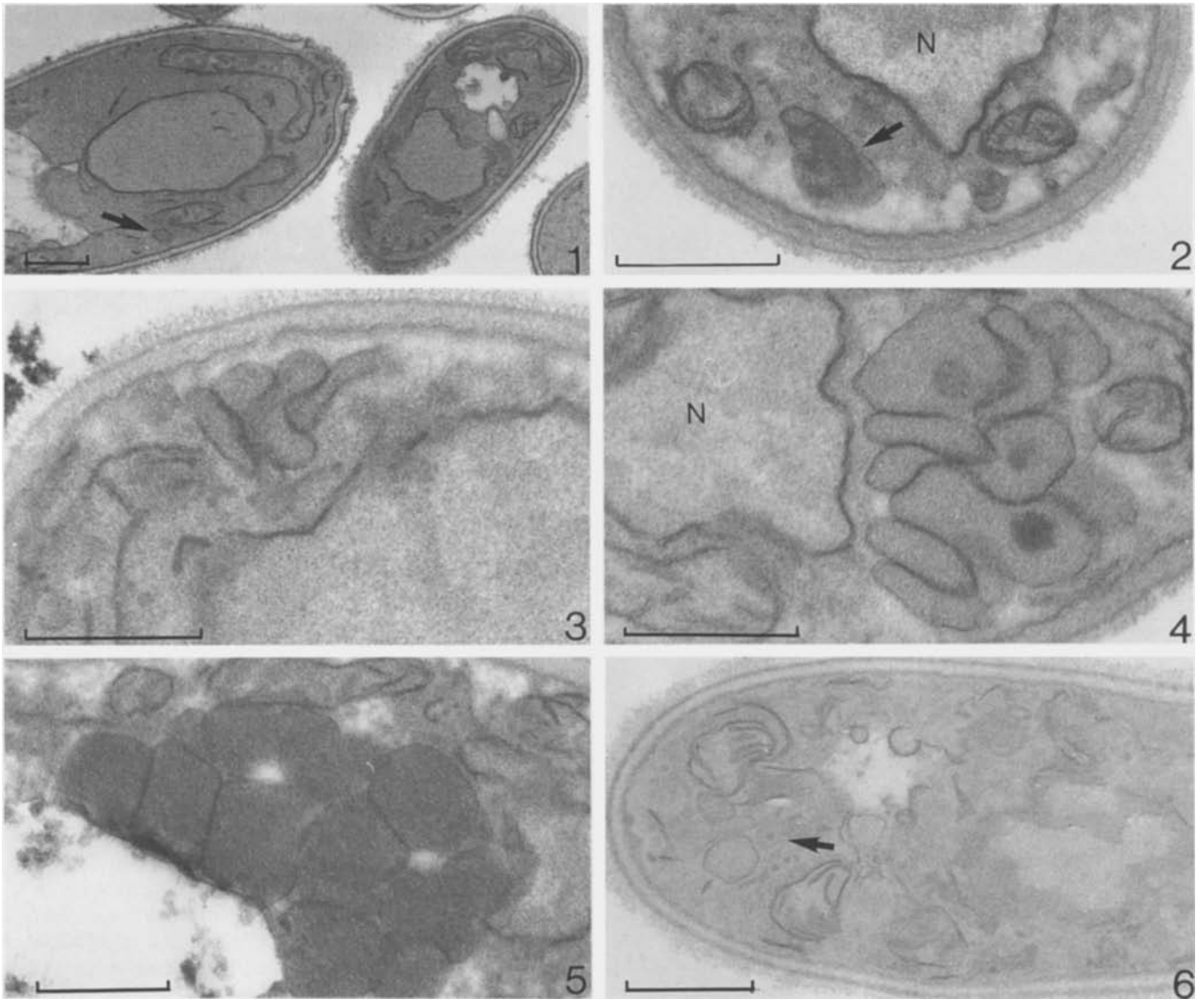
Growth and enzyme activities

After transfer of glucose/ammonium sulphate-grown cells of *Candida boidinii* into media containing 0.6% (w/v) D-alanine or 0.1% (w/v) oleic acid, growth started after a short lag of 1–2 h with growth rates of $\mu = 0.20\ h^{-1}$ (for D-alanine) and $\mu = 0.43\ h^{-1}$ (for oleic acid). Growth was not supported by propionate, ethylamine or uric acid respectively, although the two latter substrates could be utilized as nitrogen sources by the organism. In Table 1 the activities of various peroxisomal enzymes as they were present in crude extracts of cells from the mid-exponential growth phase on both D-alanine and oleic acid, are summarized. As is evident from this Table, the shift from glucose to D-alanine resulted in enhanced levels of catalase, D-amino acid oxidase and the two key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase; of the β-oxidation enzymes in particular the thiolase activity had increased. Induction of β-oxidation enzymes, together with catalase, also occurred after the shift of cells into oleic acid-containing media (Table 1). Alcohol oxidase activities could not be detected at these stages of growth and were solely observed after the cultures had entered the stationary phase of growth (data not shown).

Electron microscopy

Cells of *C. boidinii* from the mid-exponential growth phase on glucose, which were used as inoculum for cultures on oleic acid or D-alanine, characteristically contain a few small peroxisomes (Fig. 1). After the shift of cells to D-alanine as combined carbon and nitrogen source, the organelles rapidly grew and divided into many small, elongated organelles which were tightly interwoven. In the initial hours after the shift this interwaving pattern could only be clearly resolved by analysis of serial sections (not shown). Subsequently the individual organelles rapidly grew in size and remained in the interwoven state. Different stages of their development are shown in Figs. 2–4. As a result generally one or two clusters of peroxisomes developed in the cells which could be composed of up to 15–25 individual organelles (determined by analysis of series of sections). Upon maturation of these organelles a dense core developed in their matrix (Fig. 4); crystalline structures were not observed. Cytochemically the activities of catalase and D-amino acid oxidase were demonstrated to be associated to these organelles (Fig. 7A, B). The relative volume fraction of peroxisomes in batch cultures of *C. boidinii* in the late exponential growth phase amounted 6.3% of the cytoplasmic volume. Their volume increased considerably when the cells entered the stationary phase of growth due to derepression of alcohol oxidase synthesis. Import of this protein also had effects on the size and the shape of the individual organelles (Fig. 5) which were considerably enlarged and now assumed the more cubic shape, characteristic for peroxisomes in methanol-grown cells (Veenhuis and Goodman, unpublished data).

Figure 6 shows a typical example of cells grown on glucose in the presence of D-alanine as sole nitrogen source. Under these conditions relatively few per-



All micrographs were taken of whole cells of *Candida boidinii* fixed with $KMnO_4$ unless otherwise indicated. *Abbreviations:* *L*, lipid droplet; *N*, nucleus; *P*, peroxisome; *V*, vacuole. The marker represents 0.5 μm

Fig. 1–4. Different stages of peroxisomal development after a shift of cells from glucose to D-alanine as the combined carbon and nitrogen source. The inoculum (glucose-grown) cells contained few peroxisomes (Fig. 1; *arrow*). These develop in clusters of thightly interwoven peroxisomes. Typical examples of these, present in cells harvested at 5 h, 10 h and 20 h after the shift to D-alanine, are shown in Figs. 2, 3 and 4, respectively. After 20 h of cultivation the organelles contained a dense core (Fig. 4)

Fig. 5. Proliferation of peroxisomes in D-alanine-grown cells from the stationary phase of growth (30 h). Dense cores are no longer visible; individual organelles are cuboid of shape

Fig. 6. Proliferation of peroxisomes (*arrow*) in cells grown on glucose in the presence of D-alanine as the sole nitrogen source

oxisomes are present, often associated in one or two loosely bound clusters. In the late exponential growth phase their relative volume fraction amounted 0.8% of the cytoplasmic volume. The typical interwoven appearance of peroxisomes, present in cells grown on D-alanine as combined carbon and nitrogen source was seldomly observed.

In Fig. 8 the proliferation of peroxisomes in oleic acid-grown cells is depicted. Kinetic studies indicated that the development of these organelles occurred by the same mechanism as described above for D-alanine-grown cells. The major morphological difference compared to D-alanine-induced peroxisomes pertained to the shape and subcellular distribution of the organelles. During early exponential growth on oleic acid the developing organelles were predominantly elongated of shape which became more rounded upon maturation and lacked dense cores or crystalline inclusions. In the late exponential growth phase they were present in small clusters of few loosely bound organelles or as single organelles randomly distributed throughout the cytosol and made up to 12.8% of the cytoplasmic volume. Similar to D-alanine-grown cells the shape and volume fraction of the peroxisomes increased again when the cultures entered the stationary phase of growth due to the synthesis and import of alcohol oxidase protein.

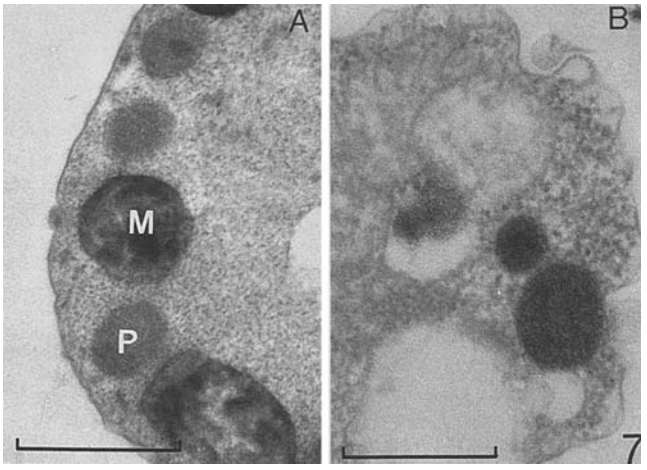


Fig. 7 A, B. Cytochemical demonstration of catalase activity in the matrix of peroxisomes (**A**) in oleic acid-grown cells (**A**; spheroplast, DAB/H_2O_2; glutaraldehyde-$OsO_4/K_2Cr_2O_7$). **B** shows positively stained peroxisomes after incubation of spheroplasts, prepared from D-alanine-grown cells, with $CeCl_3$ and D-alanine for the detection of D-amino acid oxidase activity (formaldehyde-$OsO_4/K_2Cr_2O_7$)

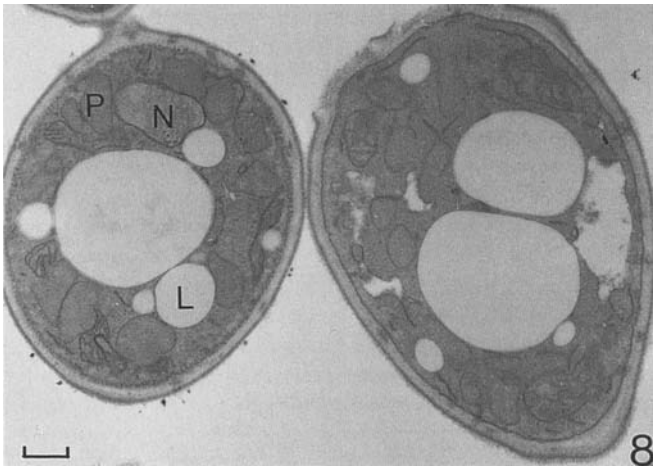


Fig. 8. Proliferation of peroxisomes in oleic acid-grown cells after 15 h of cultivation

Fractionation experiments

The subcellular localization of different enzymes involved in D-alanine- or oleic acid-metabolism was further studied by cell fractionation methods. The $30000 \times g$ pellet from lysates of protoplasts of D-alanine-grown cells of *C. boidinii* was subjected to sucrose gradient centrifugation. Analysis of the different fractions revealed that bulk of the catalase activity sedimented in a protein peak at approximately 48% sucrose, together with D-amino acid oxidase. Activity of the mitochondrial marker enzyme cytochrome C oxidase was mainly present in a protein peak at approximately 42% sucrose. The activities of the different enzymes measured in both peak fractions are summarized in Table 2.

Identical methods were applied for the fractionation of cells grown on oleic acid. However, after sucrose density centrifugation of the $30000 \times g$ pellet only a single protein peak was observed at approximately 45% sucrose. In this peak fraction catalase activity had sedimented, together with the β-oxidation enzymes (bifunctional enzyme and the thiolase activity); the mitochondrial contamination in this fraction was, judged from the cytochrome C oxidase activity, very low (Table 2). In these gradients no distinct cytochrome C oxidase peak fraction was observed. These results indicate that bulk of the mitochondria were removed during the differential centrifugation steps of homogenates of oleic acid-grown cells. The high purity of the peroxisomal peak fractions of both D-alanine and oleic acid-grown cells was confirmed by electron microscopy (not shown).

Discussion

In the present work we have shown that the methylotrophic yeast *Candida boidinii* can utilize both D-alanine and oleic acid as sole carbon sources for growth whereas both compounds induce pronounced peroxisomal proliferation. In this respect the organism differs from another methylotroph, namely *Hansenula polymorpha* which cannot grow on oleic acid (M. Veenhuis and W. H. Kunau, unpublished results) and can utilize D-alanine only as a nitrogen source (Veenhuis and Harder 1987). Growth of *C. boidinii* on D-alanine as the combined carbon and nitrogen source was accompanied by the accumulation of ammonium ions in the culture fluid (data not shown). This is the expected result from growth on this amino acid due to its relative high nitrogen ratio (C/N = 3) compared to cell material (C/N = 7). This indicates that the synthesis of D-amino acid oxidase in *C. boidinii* is not repressed by ammonium ions. Similar mechanisms have been observed for the regulation of peroxisomal amine oxidase and uricase in those yeasts species which are able to grow on ethylamine (Veenhuis et al. 1986) or uric acid (Veenhuis et al. 1985) as combined carbon and nitrogen source. The pattern of peroxisomal proliferation in D-alanine-grown *C. boidinii* is consistent with results obtained with other yeasts, capable to utilize certain compounds as combined carbon and nitrogen source (e.g. ethylamine or uric acid) in that highest proliferation invariably is observed in those cases where such compounds are used as carbon source (Veenhuis and Harder 1987; Veenhuis et al. 1985, 1986; Zwart et al. 1983).

Peroxisomes in D-alanine-grown *C. boidinii* were enriched in D-amino acid oxidase activity which is the key enzyme in the metabolism of this compound; mediated by this enzyme D-alanine is converted into pyruvate (which can enter cell intermediary metabolism for both synthesis of biomass and energy transduction; Zwart 1983), ammonia [which may be assimilated into amino acids via (NADP)glutamate dehydrogenase] (Veenhuis et al. 1985) and H_2O_2 (decomposed by catalase). We have no possible clue for the characteristical interwoven appearance of the organelles with respect to their metabolic function. This morphology most probably is not a prerequisite for an efficient D-alanine metabolism since it

Table 2. Enzyme distribution in cytochrome C oxidase (A) and catalase peak fractions (B) after discontinuous sucrose gradient centrifugation of microbody-enriched fractions (30,000 × *g* fractions), obtained by differential centrifugation of homogenized *Candida boidinii* protoplasts. Protoplasts were prepared from cells from the mid-exponential growth phase on D-alanine or oleic acid (A_{663} = 1.2)

	Growth substrate						
	D-Alanine			Oleic acid			
Enzymes	D-aox	cat	cytcox	bfe	thiol	cat	cytcox
Peak fraction A	70	60	1880	–	–	–	–
Peak fraction B	300	253	300	373	368	56	51

Catalase is expressed as *ΔE240/min · mg protein, other activities as U · 10^{-3}*/ml. Abbreviations: D-aox, D-amino acid oxidase; cat, catalase; cytcox, cytochrome C oxidase; bfe, bifunctional enzyme; thiol, 3-oxoacyl CoA thiolase

was not observed in D-alanine-grown *C. utilis* (Zwart et al. 1983), where the organelles were randomly distributed throughout the cytoplasm. By analogy, clustered peroxisomes are found in uric acid-grown *C. famata* but not in *Trichosporon cutaneum* strains, growing under the same conditions (Veenhuis et al. 1985). Therefore, the final morphology of peroxisomes in yeasts is probably not solely related to growth conditions but also appears to be species-dependent. Furthermore, the mechanisms involved in maintaining these organelles in a certain shape and/or conformation are fully unknown.

In conclusion, we have shown that carbon sources, other than methanol, the metabolism of which is associated with peroxisome-borne enzymes, can be utilized by *C. boidinii*. This offers the opportunity to further manipulate the proliferation of these organelles and their enzymic contents. Now that antibodies are available against different matrix and integral membrane proteins (Goodman et al. 1986) this greatly benefits current and future studies on the fundamental mechanisms involved in biogenesis/assemblage and metabolic functioning of these organelles.

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References

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

Dijken JP van, Veenhuis M, Vermeulen CA, Harder W (1975) Cytochemical localization of catalase activity in methanol grown *Hansenula polymorpha*. Arch Microbiol 105:216–267

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

Dixon G, Kornberg HL (1959) Assay methods for key enzymes of the glyoxylate cycle. Biochem J 72:3–9

Douma AC, Veenhuis M, de Koning W, Evers M, Harder W (1985) Dihydroxyacetone synthase is localized in the peroxisomal matrix of methanol grown *Hansenula polymorpha*. Arch Microbiol 143:237–243

Fukui S, Tanaka A (1979) Yeast peroxisomes. Trends Biochem Sci 4:246–249

Goodman JM, Scott CW, Donahue PN, Atherton JP (1984) Alcohol oxidase assembles post-translationally into the peroxisome of *Candida boidinii*. J Biol Chem 259:8485–8493

Goodman JM, Maher J, Silver PA, Pacifico A, Sanders S (1986) The membrane proteins of the methanol induced peroxisome of *Candida boidinii*. Biol Chem 261:3464–3468

Kionka Chr, Kunau WH (1985) Inducible β-oxidation pathway in *Neurospora crassa*. J Bacteriol 161:153

Kunau WH, Kionka Chr, Ledebur A, Mateblowski M, Moreno de la Garza M, Schultz-Borchard U, Thieringer R, Veenhuis M (1987) β-Oxidation systems in eukaryotic microorganisms. In: Fahimi HD, Sies H (eds) Peroxisomes in biology and medicine. Springer, Berlin Heidelberg New York, pp 128–140

Lück H (1963) Catalase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Academic Press, London New York, pp 885–894

Veenhuis M, van Dijken JP, Harder W (1976) Cytochemical studies on the localization of methanol oxidase and other oxidases in peroxisomes of methanol-grown *Hansenula polymorpha*. Arch Microbiol 111:123–125

Veenhuis M, Keizer I, Harder W (1979) Characterization of peroxisomes in glucose-grown *Hansenula polymorpha* and their development after the transfer of cells into methanol-containing media. Arch Microbiol 120:167–175

Veenhuis M, Hoogkamer-te Niet MC, Middelhoven WJ (1985) Biogenesis and metabolic significance of microbodies in urate-utilizing yeasts. Ant van Leeuwenhoek J Microbiol Serol 51:33–43

Veenhuis M, van der Klei IJ, Harder W (1986) Physiological role of microbodies in the yeast *Trichosporon cutaneum* during growth on ethylamine as the source of energy, carbon and nitrogen. Arch Microbiol 145:39–50

Veenhuis M, Harder W (1987) Peroxisomes in Biology and Medicine. In: Fahimi HD, Sies H (eds) Peroxisomes in biology and medicine. Springer, Berlin Heidelberg New York, pp 436–457

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) Yeast microbodies. Their substructure, biogenesis and turnover in relation to environmental conditions. In: Rose AH (ed) Yeast 5 (special issue) pp 517–524

Verduyn C, van Dijken JP, Scheffers WA (1984) Colorimetric alcohol assays with alcohol oxidase. J Microbiol Methods 2:15–25

Zwart KB (1983) Metabolic significance of microbodies in the yeasts *Candida utilis* and *Hansenula polymorpha*. Ph. D. Thesis, University of Groningen, The Netherlands

Zwart KB, Overmars EH, Harder W (1983) The role of peroxisomes in the metabolism of D-alanine in the yeast *Candida utilis*. FEMS Microbiol Lett 19:225–231