Weast microbodies; occurrence, proliferation and metabolic significance.
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Eukaryotic cells contain a number of distinct subcellular compartments (organelles). Each type is involved in a number of specific metabolic functions and consequently harbours its own characteristic group of enzymes and proteins. Several of these organelles have been – and still are – objects of extensive research (i.e. mitochondria and vacuoles) and much is already known about their significance in cellular metabolism and about their biogenesis. However, the current knowledge of microbodies, which were only discovered recently as a separate class of ubiquitous subcellular organelles, is still fragmentary. Microbodies are morphologically simple in construction: they are generally spherical of shape, measure from 0.2-1.5 μm, lack ribosomes and contain a finely granular proteinaceous matrix surrounded by a single membrane of 7-8 nm. Biochemically they are defined by the presence of catalase in their matrix. In addition a variety of other enzyme proteins may be present depending on the type of organism and their developmental stage and/or environmental conditions. Characteristic for developing microbodies in various eukaryotic cells – including yeasts – is their intimate association with the endoplasmic reticulum (ER). The classical concept of microbody biogenesis was largely based on these observations and proposed that microbodies develop from the smooth ER by budding.

In yeasts the development of microbodies can be induced by a number of organic growth substrates; under these conditions the organelles are thought to be either involved in the oxidative metabolism or assimilation of these substrates. This thesis describes a detailed ultrastructural and biochemical investigation into the mechanisms involved in microbody biogenesis, substructure and proliferation, as well as into their metabolic significance in relation to various conditions of growth.

The study was started with the yeast Hansenula polymorpha in which microbodies are abundantly present during growth on methanol as the sole source of carbon and energy.
Microbodies play an indispensable role in the metabolism of this C₁-compound since they harbour the enzymes involved in the initial oxidation of methanol, namely alcohol oxidase and catalase (chapter II). On the other hand, during growth of the cells on glucose activities of these enzymes were absent or extremely low. At the subcellular level such cells generally contained a single small microbody (0.1-0.3 μm) localized in the vicinity of the cell membrane and closely associated with strands of ER. Biochemically these organelles are characterized by the presence of very low activities of catalase, D-amino acid oxidase, uricase and L-α-hydroxy acid oxidase. After a shift of glucose-grown cells to media containing methanol as the sole carbon source, alcohol oxidase and catalase are rapidly synthesized. The results, discussed in chapter II, clearly demonstrate that these newly synthesized enzymes are imported into the small microbodies originally present in the (glucose-grown) inoculum cells. Growth of the organelles, resulting from this protein import, was paralleled by the formation of crystalline cores in the microbody matrix, whose development was completely dependent on the presence of alcohol oxidase protein. During prolonged incubation of cells in methanol, the number of microbodies gradually increased by the separation of small organelles from mature ones; the latter organelles measured up to 1.0 μm and contained large crystalline inclusions.

The mode of microbody development described above was confirmed in further studies with H. polymorpha during vegetative and generative reproduction of cells on methanol (Chapter III-IV). Similarly, microbodies present in developing buds originated from mature organelles present in the mothercell. Fission generally took place in the neck between mothercell and bud. These studies also revealed that both in ascospores and vegetative cells, apart from the enzymatic composition, also the ultimate shape, size, substructure and volume fraction of microbodies is largely determined by growth conditions. In methanol-limited chemostat-grown cells invariably completely crystalline organelles were found which, under appropriate conditions (D=0.03 h⁻¹), may occupy up to 80% of the cytoplasmic volume. Chapter V describes
that these crystalloids are composed of alcohol oxidase protein. A three-dimensional model of this crystalloid is presented, which also explains the observed free mobility of other microbody-matrix proteins.

Chapter VI describes the turnover of microbodies in methanol-grown *H. polymorpha* under conditions where their major matrix proteins are no longer required for growth. Our results indicated that the degree to which the microbody population, originally present in the cells, is degraded, is dependent on the extent of repression of alcohol oxidase synthesis in the new environment. Full repression of alcohol oxidase synthesis by adding excess glucose or ethanol to the incubation media led to the total degradation of the alcohol oxidase-containing microbodies. The organelles were degraded individually by an autophagic process, resulting in the complete turnover of their matrix proteins. This process was initiated by the sequestration of the organelles from the cytosol by a number of membranous layers. The vacuole played a crucial role in the degradation process since it administered the hydrolytic enzymes required for proteolysis to these compartments. The degradation was found to be an irreversible, energy dependent and - above all - fast process; individual organelles could be fully degraded within a time interval of 30 min. Not all the organelles present in one cell finally disappeared. Depending on the composition of the new environment one or more organelles remained, which developed from the alcohol oxidase-containing microbodies but whose enzymic composition now was a reflection of the new environment.

Chapter 7 and 8 describe examples of microbodies involved in the metabolism of both the carbon and the nitrogen source. The first example was encountered in newly isolated strains of *Candida famata* and *Trichosporon cutaneum* capable of utilizing uric acid as the combined carbon- and nitrogen source (chapter VII). Growth at the expense of uric acid was associated with the development of many microbodies in the cells which contained high levels of urate oxidase and catalase. The second example was observed in a newly isolated strain of *Trichosporon cutaneum* X4 that could grow on
cells contained microbodies that harboured catalase, as was shown cytochemically. In both organisms the urate oxidase- or amine oxidase-containing microbodies developed from organelles present in the inoculum cells. It must be stressed that growth of the above organisms on uric acid or ethylamine provide unique examples of the proliferation and physiological function of yeast microbodies, since enzymes other than microbody-borne urate oxidase and amine oxidase which are involved in the initial metabolism of these substrates, were not detected. Therefore, in these organisms all the carbon and nitrogen required for growth flows via microbodies where enzymes are present that are crucial for the initial metabolism of these combined carbon and nitrogen sources.

Utilization of uric acid or ethylamine as carbon source inevitably leads to the excretion of ammonium into the cultivation medium as a result of the low carbon (C) over nitrogen (N) ratio's in both substrates compared to cell material (C:N uric acid = 5:4; C:N ethylamine = 2:1 versus cell material C:N = 7:1). Utilization of these substrates as C-source could be explained by the finding that the synthesis of both amine oxidase and uric acid oxidase was insensitive to repression by free ammonium ions. Our results indicated that the synthesis of these enzymes was regulated by induction.

The physiological significance of the microbodies in ethylamine-grown *T. cutaneum* X4 was further investigated by fractionation studies. Intact protoplasts were gently homogenized and subsequently fractionated by means of differential and sucrose gradient centrifugation. The results showed that the isolation of intact microbodies was critically dependent on the pH, composition and osmotic strength of the homogenisation medium. Analysis of the different fractions obtained indicated that microbodies of ethylamine grown *T. cutaneum* X4 contained, besides catalase and amine oxidase, key enzymes of the glyoxylate cycle namely isocitrate lyase and malate synthase, together with malate dehydrogenase, glutamate:oxaloacetate aminotransferase and an enzyme not encountered before in microbodies, namely
(NAD-dependent) glutamate dehydrogenase. This subcellular localization of these enzymes requires interlocking of the metabolic activities of microbodies and mitochondria for balanced growth at the expense of ethylamine. The microbodies produce aspartate which serves as a major cytosolic intermediate for carbon assimilation.

In chapter 9 the presence and proliferation of microbodies in *Saccharomyces cerevisiae* (baker's yeast) is described. In this industrially important yeast microbodies are generally present in very small numbers and consequently were only poorly characterized. Attempts to induce their proliferation had so far failed. This general behaviour of baker's yeast is confirmed by the results discussed in chapter 9. Microbody proliferation was not observed during incubation of cells under a variety of growth conditions that are known to induce microbody proliferation in other yeasts with only one exception, namely during growth on oleic acid. Marked proliferation of microbodies was observed after a shift of cells from glucose to media containing oleic acid. This was associated with induction of activities of β-oxidation enzymes and catalase. Ultrastructural investigations carried out at different time intervals suggested that also in baker's yeast microbodies developed from already existing organelles by growth and division.

The results discussed in this thesis support the general hypothesis that at least in aerobically-grown yeasts microbodies are invariably present irrespective of the cultivation conditions. Their function can become important for the metabolism of certain carbon and/or nitrogen sources by import of several substrate-specific enzymes. During adaptation of cells to a different environment newly formed microbodies (peroxisomes, glyoxisomes or functionally intermediate forms) develop from organelles already present in the cell prior to the transition. Their ultimate shape, volume fraction, substructure and enzymatic composition is completely dependent upon environmental conditions.