Immunocytochemical evidence for the acidic nature of peroxisomes in methylotrophic yeasts

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The possible acidic nature of the peroxisomal matrix present in intact yeast cells was studied immunocytochemically, using the weak base DAMP as a probe. Spheroplasts of methanol-grown *Candida boidinii* and *Hansenula polymorpha* were regenerated and incubated with DAMP. After immunogold labelling, using antibodies against DAMP, a specific accumulation of gold particles was observed on the peroxisomal profiles. This labelling was absent in controls, performed in the presence of ionophores or chloroquine. These results support earlier observations, that in intact cells a pH-gradient exists across the peroxisomal membrane. Experiments, carried out on osmotically swollen spheroplasts indicated that maintenance of this pH-gradient is strongly related to the cell's integrity.

Peroxisome; Proton gradient; Immunocytochemistry; (*Candida boidinii; Hansenula polymorpha*)

1. INTRODUCTION

In yeasts, the proliferation and enzymic composition of microbodies (peroxisomes, glyoxysomes) is largely dependent on growth conditions [1]. Precursors of matrix proteins are made on free polysomes and imported post-translationally into their target organelles where assembly and activation takes place [2]. However, still little is known on the molecular mechanisms involved in protein import/assembly and microbody functioning. Recently Nicolay et al. [3] provided evidence that a proton gradient exists across the peroxisomal membrane in yeasts. This evidence was based on the finding that the peroxisomal matrix in intact cells is acidic (pH 5.8–6.0) compared to the cytosol (pH 7.1). Since maintenance of a certain energy status may be of significance for different aspects of both microbody development [4] and/or functioning (e.g. in that it functions as a driving force for transport processes across the peroxisomal membrane), we sought additional evidence for their acidic nature in vivo. For this purpose we employed a recently developed immunocytochemical method, which is based on the accumulation of the weak base 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP) in acidic compartments [5]. The results of this study are presented in this paper.

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2. MATERIALS AND METHODS

2.1. *Micro-organisms and growth*

Candida boidinii ATCC 32195 and *Hansenula polymorpha* de Morais et Maya CBS 4732 were grown in batch cultures in the mineral medium as described before [6] at 30 and 37°C, respectively. *H. polymorpha* was also grown in a carbon-limited continuous culture in mineral medium [7] at a dilution rate (D) of 0.07 h^{-1}, using 1% (w/v) methanol as the source of carbon and energy.

2.2. *Preparation and regeneration of spheroplasts*

Spheroplasts were prepared by treatment of suspensions of intact cells with zymolyase [8]. Prior to the incubation with DAMP the spheroplasts were regenerated in an osmotically stabilized mineral medium (adjusted to pH 7.2) for 3 h in shake flasks (50 rpm) at 30 or 37°C, respectively. As osmotic stabilizer, 1.5–2 M sorbitol was added; the media were supplemented with 0.5% (w/v) methanol (in the case of *C. boidinii*) or 30 mM Na-formate (in the case of *H. polymorpha*) as carbon and/or energy source.

2.3. *Incubation with DAMP*

Regenerated spheroplasts were incubated for 1 h with 35 μM DAMP (Oxford Biomedical Research), which was added directly to the medium. After incubation, the spheroplasts were collected by centrifugation, carefully resuspended in fresh osmotically stabilized complete mineral medium and incubated for 30 min to remove excess unprotonated DAMP. As controls spheroplasts, which had been incubated with DAMP, were subsequently exposed to nigericine and valinomycine (2 μM each) or to nigericine only for 15 min. As another control, spheroplasts were incubated with chloroquine (10 mM final concentration) for 1 h prior to or after the incubation with DAMP.

2.4. *Immunocytochemistry*

After the incubations, spheroplasts were fixed by the addition of glutaraldehyde (final concentration 2% (w/v)) to the cell suspensions. After fixation for 1 h at 0°C, the samples were pelleted and embedded in Lowicryl K4M [9]. Immunocytochemical experiments

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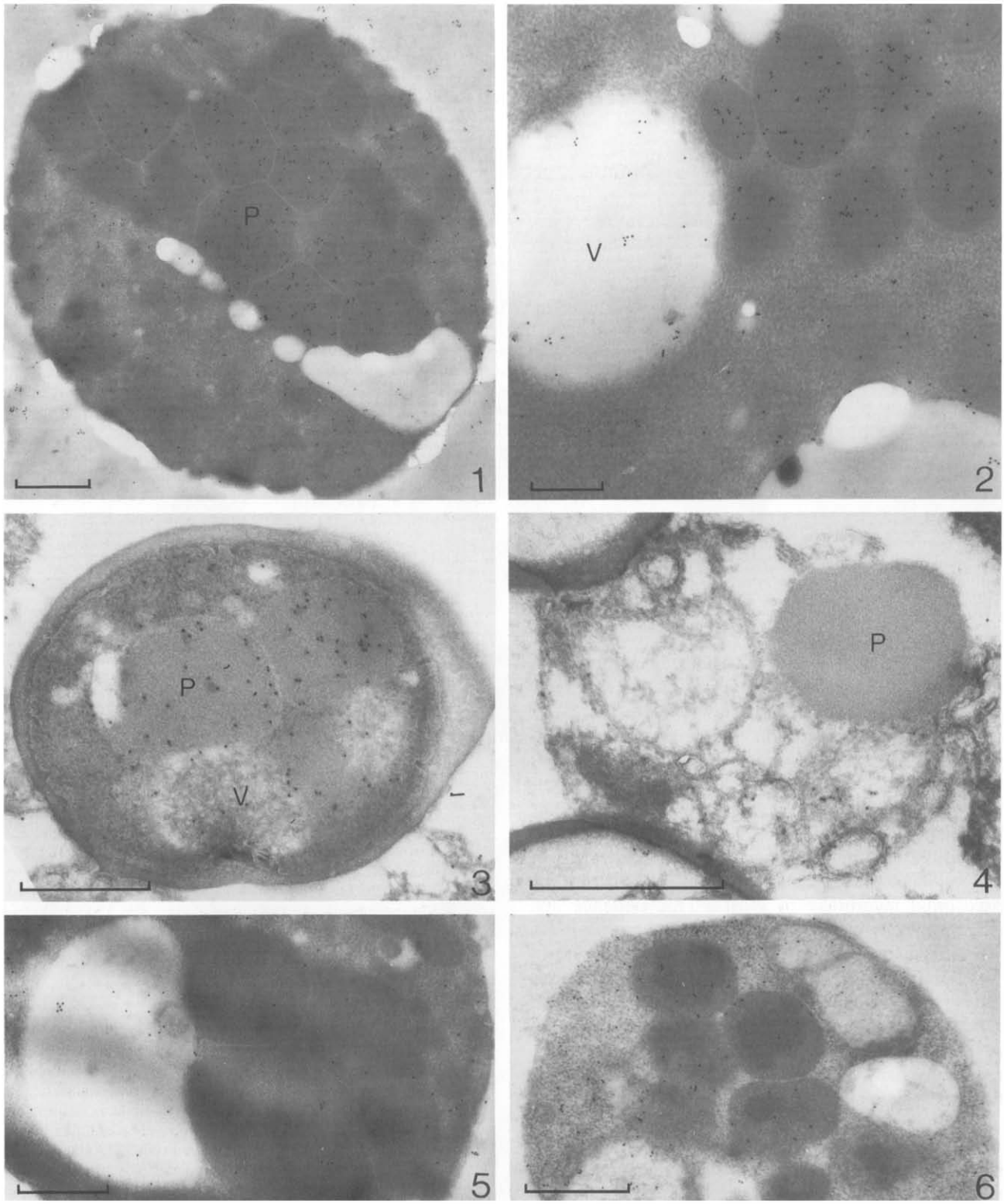

Figs 1–6. Immunocytochemical demonstration of the accumulation of DAMP in acidic subcellular compartments of yeasts. Abbreviations: V, vacuole; P, peroxisome. Marker bar = 0.5 μm.

Figs 1,2. Accumulation of gold particles on peroxisomal profiles of *C. boidinii* (fig.1). Similar labelling patterns were observed on small developing peroxisomes (fig.2).

Figs 3,4. Labelling of peroxisomes in methanol-limited spheroplasts of *H. polymorpha* (fig.3). In osmotically disrupted peroxisomes no labelling is observed (fig.4).

Figs 5,6. Control experiments, showing labelling of peroxisomes after incubations in the presence of ionophores (fig.5) or chloroquine (fig.6).

were performed on ultrathin sections using anti-dinitrophenol (α-DNP) monoclonal antibodies (kindly provided by Dr R.G.W. Anderson, Southwestern Medical Center at Dallas, USA) and protein A/gold [10].

3. RESULTS AND DISCUSSION

Initial experiments in which DAMP was used for the detection of acidic compartments in intact yeasts gave highly irreproducible results. This was most probably due to a poor penetration of DAMP through the relatively thick cell walls of these organisms. Therefore, spheroplasts were used in further studies. In immunocytochemical experiments, performed on ultrathin sections of spheroplasts from methanol-grown *C. boidinii* with specific antibodies against DAMP and protein A/gold, abundant labelling was observed on the peroxisomal profiles compared to the cytosol (fig.1), indicating that DAMP had actually accumulated in these organelles. This labelling was independent of the developmental stage of the organelles (fig.2). Comparable labelling patterns were found on the vacuoles. Essentially similar results were obtained with methanol-grown cells of *H. polymorpha* (fig.3). Such intensified labelling was not observed on peroxisomal profiles of partly disrupted spheroplasts nor in peroxisomes released from spheroplasts (fig.4), suggesting that the energy status of the organelles must be strongly related to the cell's integrity. In control experiments conducted in the presence of nigericin or nigericin plus valinomycin, specific labelling of peroxisomes was not observed; in both samples labelling of the organelles did not or hardly exceeded the cytosolic background labelling (fig.5). Essentially similar results were obtained in experiments performed in the presence of chloroquine (fig.6).

Based on ^{31}P-NMR studies, Nicolay et al. [3] provided the first evidence that the peroxisomes in intact yeast cells are acidic in nature. Our present immunocytochemical data support these results. The peroxisomal proton gradient, or proton-motive force, is most probably generated and maintained by a H^+-ATPase located on the peroxisomal membrane [11,12]. Evidence is now accumulating that ATPases are also associated with peroxisomes of other organisms, e.g. rat liver [13]; this poses the question whether the observed acidity of peroxisomes in yeast is a general property of these organelles.

Proton-motive forces across organellar membranes in eukaryotic cells may play a role in different processes. They have been shown to be involved in the translocation of proteins [14] as well as transport of enzyme substrates and/or products across these membranes [15]. The proton-motive force across yeast peroxisomal membranes may serve similar purposes. However, a major impediment in studies on the mechanisms involved in microbody biogenesis and functioning is the discrepancy which still exists between the in vivo and in vitro situation. In vitro peroxisomes, including yeast peroxisomes, are invariably leaky [16]; in rat liver the observed permeability is most probably caused by weakly cation-selective conductance channel proteins (porins) present in the peroxisomal membrane [17].

The finding that DAMP did not accumulate in virtually intact peroxisomes present in osmotically swollen protoplasts, confirms earlier observations [16] that the leakiness of the organelles is strongly related to the cell's integrity. Douma et al. [16] furthermore showed that the putative pore-forming protein, present in yeast peroxisomal membranes, cannot readily be closed. Therefore, an in vitro approach directed to study the possible energy-dependency of different transport processes across the peroxisomal membrane awaits elucidation of the mechanisms regulating this pore-forming protein.

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