Monitoring of peroxisome induction and degradation by flow cytometric analysis of *Hansenula polymorpha* cells grown in methanol and glucose media: cell volume, refractive index and FITC retention

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Cell refractive index has been used to monitor peroxisome behaviour in the yeast *Hansenula polymorpha* by means of flow cytometry. Peroxisomes are inducible organelles which may occupy a large fraction of the cell volume when yeast cells are growing in methanol media. These organelles harbour a catalase that decomposes the hydrogen peroxide produced in methanol oxidation by alcohol oxidase, a peroxisomal enzyme whose subunits are arranged to form a regular crystalloid. Peroxisomes undergo a degradation process mediated by vacuoles whenever they and their enzymes become metabolically redundant (e.g. during growth on glucose). Flow cytometric analyses of side scattered light (depending on cell volume, morphology and structure) and fluorescein isothiocyanate retention (due to the vacuole) were made on two wild-type strains of *H. polymorpha* during exponential growth in glucose and methanol media and during nutritional shifts from one carbon source to the other. The same parameters were also analysed for a mutant strain only partially repressed by glucose. We show that both the parameters are substrate-dependent and appear to reflect peroxisome development in the cells. The data reported correlate well with the known cytological and biochemical data, showing the possibility of using flow cytometry, a fast and sensitive technique, to analyse the dynamics of peroxisome proliferation and degradation in response to environmental as well as genetic factors.

Keywords: *Hansenula polymorpha*, peroxisomes, flow cytometry, refractive index, FITC retention

INTRODUCTION

Microbodies (peroxisomes, glyoxysomes) are organelles found in all eukaryotes. Their metabolic significance has been investigated in many organisms, making use of biochemical and genetic tools (for a comprehensive review see Borst, 1989). The discovery that their absence or functional impairment in man cause severe abnormalities (some of which are lethal) has raised considerable interest (Lazarow & Moser, 1989; Moser *et al.*, 1991).

Yeasts are ideal biological models for microbody studies, since in many species they respond to the presence of

various carbon and nitrogen sources in the medium by enlarging pre-existing microbodies (or possibly newly synthesized ones; Waterham *et al.*, 1993) and by increasing their number – a process known as microbody proliferation. This is the case observed in methylotrophic yeasts, such as the ascomycete *Hansenula polymorpha* (synonym: *Pichia angusta*), when growing in methanol-containing media (Veenhuis *et al.*, 1983; Veenhuis & Harder, 1991). In the presence of methanol, *H. polymorpha* undergoes microbody proliferation, the extent of which varies with the growth conditions. These microbodies are called peroxisomes, as they harbour a catalase that decomposes the hydrogen peroxide produced in methanol oxidation. This reaction is the first one in methanol metabolism and is carried out by an alcohol oxidase (AO), also peroxisomal, which yields formaldehyde. AO occurs as octameric molecules, with subunits arranged to form a

Abbreviations: AO, alcohol oxidase; FITC, fluorescein isothiocyanate; FL1, fluorescein isothiocyanate fluorescent light signal; FSC, forward scattered light; SSC, side scattered light.

crystalloid, i.e. a highly regular structure with cubical architecture (van der Klei *et al.*, 1991). The amount of this and other peroxisomal proteins (which include dihydroxyacetone synthase, involved in formaldehyde assimilation) largely depends on the growth conditions: up to 80% of the cell volume may be occupied by large cuboid peroxisomes (Veenhuis & Harder, 1991), and more than 30% of the total cellular protein may be AO (Giuseppin *et al.*, 1988).

In wild-type cells, peroxisome degradation takes place whenever these organelles and their enzymes become metabolically redundant, as in the case of methanol-grown cells transferred into media containing other carbon sources, such as glucose (Veenhuis *et al.*, 1983a, b). Inhibition of peroxisomal protein synthesis then occurs, together with a rapid proteolytic inactivation of pre-existing peroxisomes. This degradation involves an autophagic process mediated by the vacuole, a compartment for proteolytic enzymes where proteins to be degraded might be transported (Veenhuis *et al.*, 1978, 1983a). As a result, the peroxisome number and volume decrease, and the large crystalloids disappear, so that glucose-grown cells have small, spherical peroxisomes (Veenhuis *et al.*, 1983b).

Flow cytometry is a powerful technique which allows an accurate and sensitive analysis of both structural and functional parameters. Cell-by-cell measurements of absorbed and scattered laser light or fluorescent emissions are taken to study parameters as diverse as cell size, refractive index, protein content, redox state, enzymic activities, intracellular pH and many others (reviewed by Shapiro, 1988). To study some of these parameters (e.g. cell volume and refractive index) the populations to be analysed need not be treated, as the measurements are based on light absorbed or scattered by the cells. For other parameters, instead, the populations are treated with fluorescent probes which bind to certain cell components, enter specific metabolic compartments or react with specific cell constituents. The analyses are carried out at a rapid rate such that the distribution of the parameters in the whole population can be obtained in a very short period of time.

Here we report flow cytometric studies of wild-type and mutant *H. polymorpha* cells growing in different substrates and show that cell refractive index is substrate-dependent and appears to reflect the peroxisomal development of the single cells. The vacuole dimension, measured as fluorescein isothiocyanate (FITC) retention by the cells, also correlates well with the known cytological, enzymic and metabolic data, suggesting that flow cytometry is a valuable tool to analyse the dynamics of microbody proliferation and degradation in response to environmental as well as genetic factors.

METHODS

Strains. Three *H. polymorpha* strains were used, namely ATCC 26012 (wild-type), L1 (*leu1*, a laboratory strain derived from NCYC 495; Gleeson *et al.*, 1986), and HGR26 (*leu1*, *glr1*; L1-derived, glucose-repression mutant obtained on methanol plates

containing the glucose analogue 2-deoxyglucose; E. Berardi & C. Stanway, unpublished).

Growth experiments

General. Cells were grown in conical flasks (250 ml operative volume) till exponential phase (i.e. the growth phase in which all the bioparameters of the culture do not change, 10^6 – 10^7 cells ml^{-1}) by shaking in YEP-glucose, YEP-methanol or in a mixed YEP-glucose-methanol complete medium (1%, w/v, yeast extract; 2%, w/v, peptone; 2%, w/v, glucose and/or 2%, v/v, methanol) at 30 °C.

Induction. Approximately 5×10^7 cells growing exponentially in YEP-glucose were collected, washed in sterile H_2O and transferred to 50 ml YEP-methanol (10^6 cell ml^{-1} initial cell concentration).

Repression. Glucose (diluted from a stock solution of 20%, w/v) was added to YEP-methanol cultures growing exponentially (cell density: 10^6 ml^{-1}) to a final concentration of 2% (w/v).

FITC retention. To cells growing exponentially on different carbon sources, FITC (diluted from 1 mg ml^{-1} stock solution in 0.1 M Tris/HCl, pH 8) was added to a final concentration of $10 \mu\text{g ml}^{-1}$. Aliquots (2 ml) were then harvested, after at least 30 min, by centrifugation, washed with sorbitol buffer (140 mM KCl, 1.2 M sorbitol, 20 mM Tris/HCl, pH 7.2) and resuspended in a saline phosphate buffer (3.3 mM NaH_2PO_4 , 6.7 mM Na_2HPO_4 , 0.2 mM EDTA, 130 mM NaCl, pH 7.5) for flow cytometric analyses.

AO, cell number, cell volume and flow cytometric determinations. AO assays were done following the procedures described by van der Klei *et al.* (1990).

Cell number and cell volume distributions were determined by counting with a Coulter counter Channalyzer ZBI, as described by Martegani *et al.* (1990).

Flow cytometric analysis was performed with a Becton and Dickinson FACStar^{plus} equipped with a 2 W argon-ion laser (excitation wavelength 488 nm; laser power 200 mW) (Alberghina & Porro, 1993).

FITC-fluorescence intensity (FL1) was measured at 520 nm. The flow rate was approximately 500 cells sec^{-1} and the number of cells analysed was 30 000. Preliminary experiments were done with fluorescent beads (2 μm - and 5 μm -diameter; Polysciences).

RESULTS AND DISCUSSION

Refractive index analysis of exponentially growing cells

Side scattered light (SSC) depends on volume, morphology and, more importantly, on cell granularity. The latter depends, in turn, on the internal cellular structure (Shapiro, 1988; Salzman *et al.*, 1990). Most particularly, inclusions may influence SSC profoundly, if their refractive index is different from the cytoplasmic one. For example, Dubelaar *et al.* (1987) found significant SSC differences between cyanobacteria containing or lacking gas vacuoles. SSC monitoring of *Alcaligenes eutrophus* grown in batch cultures revealed significant changes, probably reflecting shape variations of the poly- β -hydroxybutyrate refractive bodies (Srienc *et al.*, 1984). Lymphocyte labelling with antibody-conjugated gold particles showed a tenfold increase in SSC (Bohmer & King, 1984). SSC can also be employed for quantitative

Fig. 1. FSC vs SSC values of two populations of fluorescent polystyrene microspheres with different diameters but identical refractive index: (a) 2 μm ; (b) 5 μm .

Fig. 2. Wild-type strain ATCC 26012 growing exponentially in YEP-methanol (continuous line) and in YEP-glucose (dotted line). (a) Flow cytometric determination of SSC values; (b) volume distributions made with a Coulter counter Channalyzer.

analyses. Population viability, for instance, can be studied on the basis of differences between live and dead cells (Shapiro, 1988). A great advantage of SSC is its being intrinsic to the cell, so that untreated living populations are used. This advantage is particularly useful when considering cell components, such as peroxisomes, known

Fig. 4. SSC:FL1 ratio values of each cell of the wild-type strain ATCC 26012 growing exponentially in YEP-methanol (a) and YEP-glucose (b).

to be delicate and sensitive to various treatments and to be leaky in fixed cells (Douma *et al.*, 1989).

Bearing in mind these considerations, we wanted to study *in vivo* the peroxisome dynamics in response to environmental and genetic factors. We made use of the yeast *H. polymorpha*, on the assumption that the cells might have their SSC influenced by the extent of crystalline peroxisomes.

To make SSC comparisons valid, we first showed that, in addition to varying with refractive index, SSC values are also size-dependent. We used fluorescent polystyrene microspheres with different diameters (2 μm and 5 μm) but identical refractive index. Fig. 1 relates forward

Fig. 3. SSC vs FL1 values of the wild-type strain ATCC 26012 growing exponentially in YEP-methanol (a) and YEP-glucose (b).

Fig. 5. SSC vs FL1 values after the addition of glucose (2%, w/v) to the wild-type strain ATCC 26012 growing exponentially in YEP-methanol. Samples were analysed at 0, 2 and 5 h (a, b and c, respectively).

Fig. 6. Cell volume distributions made with a Coulter counter Channalyzer referring to the samples shown in Fig. 5(a) (●), (b) (Δ) and (c) (○), respectively.

scattered light (FSC; an approximate flow cytometric measure of volume) with SSC for the two microsphere populations and shows that microspheres with identical refractive index, but bigger volume, have higher SSC values.

To investigate possible relationships between refractive index and the peroxisomal status of the cells, the wild-type *H. polymorpha* strain ATCC 26012 was grown to exponential phase in YEP-glucose and YEP-methanol. Glucose-grown cells have an SSC value comparable to methanol-grown cells (Fig. 2a). However, since methanol-grown cells are considerably smaller than glucose-grown ones (Fig. 2b), we concluded that the former cells have a refractive index which is proportionally higher. It is noteworthy that size distribution determinations of the populations were made by Coulter counter analyses, which have been shown to be more reliable than the flow cytometric ones (Shapiro, 1988).

Refractive index vs FITC retention of exponentially growing cells

Differences between cells grown in glucose and methanol media are shown more dramatically in Fig. 3. Exponentially growing cells were stained with FITC, a fluorescent compound which selectively stains the yeast vacuole (De Mesquita *et al.*, 1991). It is known that cells of

Fig. 7. SSC vs FL1 values of ATCC 26012 glucose-grown cells shifted to the methanol medium. Samples were analysed at 0, 6 and 10 h after the shift (a, b and c, respectively).

Fig. 8. Cell volume distributions made with a Coulter counter Channalyzer referring to the samples shown in Fig. 7(a) (●), (b) (Δ) and (c) (○), respectively.

H. polymorpha harbour large vacuoles when growing in glucose and small ones when growing in methanol (Veenhuis & Harder, 1991). Our data are in good agreement with these observations, since the recorded SSC vs FITC signals (FL1) of *H. polymorpha* grown separately in methanol and glucose show very different cytograms (Fig. 3). The first one (methanol; a) shows distribution with relatively low FL1 values, in accordance with small vacuoles in the cells. The second one (glucose; b) shows distributions with relatively high FL1 values, indicating high FITC retention, which is likely to reflect the overall dimension/activity of the vacuoles in the cells (De Mesquita *et al.*, 1991). The two populations are therefore easily distinguishable. Derivations of these data are shown in Fig. 4. Each histogram represents the SSC:FL1 ratio, calculated for each individual cell in the population.

Refractive index vs FITC retention of transiently growing cells

To validate our previous experimental data, we analysed the dynamics of peroxisome proliferation and repression.

Proliferation is the consequence of induction phenomena, triggered by methanol, whereas inactivation is the result of complex catabolite repression mechanisms, triggered by glucose and other carbon sources, except methanol (Veenhuis *et al.*, 1983b). In *H. polymorpha* glucose repression is epistatic to methanol induction, i.e. glucose represses peroxisome proliferation and the methanol pathway even when the inducer (methanol) is present. Thus, the addition of glucose to a population growing on methanol causes a rapid inactivation of many peroxisomal enzymes, together with a dramatic vacuole-mediated peroxisome degradation (Veenhuis *et al.*, 1978, 1983a). Fig. 5 shows typical time-course SSC vs FL1 cytograms of a repression experiment. Glucose (2%, w/v) was added to cells growing exponentially in YEP-methanol and samples were analysed at 0, 2 and 5 h. During this time, the SSC values did not vary significantly, but we have to consider that cells at time 0 are smaller than the ones taken 3 and 5 h after glucose addition, as shown in Fig. 6. FL1

Fig. 9. SSC vs FL1 values of the mutant strain HGR26. Cells were growing exponentially in YEP-glucose (a), YEP-methanol (b) and in the mixed YEP-methanol-glucose medium (c). In (d), a combination is shown of two cultures growing in glucose and in methanol media separately and mixed for the flow cytometric analysis.

values, on the other hand, increased substantially. After 5 h the distribution pattern is identical to the one previously seen for glucose-grown cells (compare Fig. 5c with Fig. 3b). The activity of AO, a peroxisomal marker enzyme, was reduced by tenfold in 3–5 h [$t = 0$: 1350; $t = 2$: 350; $t = 5$: 110 mU (mg total cell proteins) $^{-1}$]. Thus, the observed SSC vs FL1 values are substrate-dependent and appear to reflect actual decreases of pre-existing peroxisomes and enlargements of vacuoles.

As expected, an opposite time-course pattern was registered when glucose-grown populations were shifted to the methanol medium (Fig. 7). Also in this case, SSC values have to be considered according to cell volumes (Fig. 8). The activity of AO has been measured [$t = 0$: 0; $t = 6$: 120; $t = 10$: 1050 mU (mg total cell proteins) $^{-1}$].

To broaden our investigation and make our results more general, we repeated the experiments just described with two laboratory strains of *H. polymorpha*, namely L1 (*leu1*) and HGR26 (*leu1*, *glr1*). The former has wild-type peroxisome induction and repression (Gleeson *et al.*, 1986), whereas the latter is a regulatory mutant partially insensitive to glucose repression (E. Berardi & A. Stanway, unpublished).

We found that cytograms of strain L1 were similar to those of strain ATCC 26012 (data not shown), whereas mutant HGR26 showed some notable differences. Upon glucose or methanol growth, SSC vs FL1 values were as in the wild-type (Fig. 9a and Fig. 9b). However, when growth was in mixed YEP-glucose-methanol medium,

the cytogram obtained showed an intermediate pattern (Fig. 9c) which is clearly distinguishable from that obtained by the simple combination of two cultures, one from glucose and the other from methanol (Fig. 9d).

CONCLUSIONS

In this work we have observed changes in the refractive index of *H. polymorpha* yeast cells growing in different substrates and shifted from one carbon source to another.

The SSC value of each cell, which is related to cell refractive index, is substrate-dependent and reflects accurately the known peroxisomal and enzymic status. The vacuole dimension/activity, measured as FITC retention, also correlates well with the known cytological enzymic and metabolic data. Comparisons between wild-type glucose-repression strains and a regulatory mutant, partially insensitive to glucose repression, confirm that flow cytometry is a valuable tool to analyse the dynamics of microbody proliferation and degradation in response to environmental as well as genetic factors.

Furthermore, sensitivity, reproducibility and speed, together with the use of intact cell populations, are among the advantages of flow cytometry. It may thus support biochemical and cytological studies and serve investigations as diverse as mutant characterization, analysis of the dynamics of growth, production of heterologous proteins and monitoring of industrial fermentations related to the peroxisomal biology of *H. polymorpha* cells.

ACKNOWLEDGEMENTS

This project was supported by CNR, Progetto Finalizzato Biotecnologie e Biostrumentazioni, Subproject n.3 to B. M. Ranzi.

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Received 13 June 1994; revised 28 July 1994; accepted 3 August 1994.