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Assessment of the microbody luminal pH in the filamentous fungus *Penicillium chrysogenum*

Ted R. van der Lende [a,1], Pieter Breeuwer [b], Tjakko Abee [b], Wil N. Konings [a], Arnold J.M. Driessen [a,*]

[a] *Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*

[b] *Department of Food Technology and Nutritional Sciences, Food Microbiology Group, Wageningen University and Research Centre, P.O. Box 9101, 6700 HB Wageningen, The Netherlands*

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Abstract

The enzymes of the penicillin biosynthetic pathway in *Penicillium chrysogenum* are located in different subcellular compartments. Consequently, penicillin pathway precursors and the biologically active penicillins have to cross one or more membranes. The final enzymatic step that is mediated by acyltransferase takes place in a microbody. The pH of the microbody lumen in penicillin producing cells has been determined with fluorescent probes and mutants of the green fluorescent protein and found to be slightly alkaline.

Keywords: Organelle; Microbody; Peroxisome; Vacuole; Penicillin; *Penicillium chrysogenum*

1. Introduction

Compartmentalization of metabolic reactions in subcellular compartments is observed in many organisms. This compartmentalization facilitates a spatial separation of reactions thus enabling different reaction conditions to exist simultaneously in a cell. The ascomycetous filamentous fungus *Penicillium chrysogenum* is well known for its capacity to produce the β-lactam antibiotic penicillin [1]. Penicillin biosynthesis is partially compartmentalized (for review see [2]). The enzyme acyl-coenzyme A:isopenicillin N (acyl-CoA:IPN) acyltransferase (IAT) [3–5] is localized in peroxisomes (also called microbodies) [6–8], and catalyzes the exchange of the L-α aminoadipic group of isopenicillin N for a more hydrophobic group to yield biologically active penicillins (e.g., phenylacetic acid in case of penicillin G). In addition, acyl-CoA ligase (PCL) (International patent WO97/02349) that activates phenylacetic acid (PA) to PA-CoA may be located in these peroxisomes. PCL and IAT both harbor a peroxisomal type 1 targeting signal (PTS1) [9,10] at the C-terminus, i.e., Ser-Lys-Ile (International patent WO97/02349) and Ala–Arg–Leu [11], respectively. In the case of IAT, the ARL se-

* Corresponding author. Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands. Fax: +31-50-3632154.

E-mail address: a.j.m.driessen@biol.rug.nl (A.J.M. Driessen).

[1] Present address: Department of Applied Microbiology and Gene Technology, TNO Nutrition and Food Research, P.O. Box 360, 3700 AJ Zeist, The Netherlands.

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quence indeed has been shown to target the protein to the microbody [6–8].

In filamentous fungi, microbodies have been shown to be involved in diverse processes like β-oxidation of fatty acids in *Neurospora crassa* [12–14] and *Aspergillus nidulans* [15,16], karyogamy in *Podospora anserina* [17], and penicillin production in *P. chrysogenum* [6–8]. However, most of our current knowledge about these organelles in fungi is based on studies on yeasts and mammals [18–20]. In terms of flux control of metabolites and efficiency of the penicillin biosynthetic pathway, compartmentalization of this pathway may be a significant factor. Firstly, within such a compartment, the ionic strength, pH and redox conditions may differ from that in the cytosol. Secondly, as a result of metabolic energy-coupled transport, the concentration of metabolites within the microbody may differ by several orders of magnitude from those in the cytosol. This may optimize enzymatic reactions within this compartment and minimize potential substrate-induced inhibition of down- and upstream enzymatic reactions that are located in the cytosol.

Microbodies of the methylotrophic yeast *Hansenula polymorpha* have been reported to be acidic, i.e., pH 5.8–6.0 [21,22], whereas the pH in the lumen of the peroxisomes of the mammalian fibroblast cells has been found to be alkaline, i.e., in the range of pH 8.5–9.0 [23]. Since two key enzymes of the penicillin biosynthetic pathway that are localized in the microbody lumen are inactive at pH values below 6.0 [3,4] (International patent WO97/02349), we have investigated the pH value of the microbody lumen in living cells of *P. chrysogenum*. For this purpose, the enhanced yellow fluorescent protein (eYFP) was fused at its C-terminus with a PTS1 signal, and targeted to the microbody to monitor the luminal pH.

2. Materials and methods

2.1. Fungal strains, plasmids and growth conditions

Escherichia coli DH5α [Φ80Δ*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hdsR17* (r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*] was used for plasmid transformations. The strains used were *P. chrysogenum* Wisconsin 54-1255 (Wis54-1255) [24] producing a relatively low amount of penicillin and DS04825, a strain that produces relatively high amounts of penicillin [25]. *P. chrysogenum* mycelia were grown for 1–3 days in production medium [26]. Plasmid pBluescript II KS (Stratagene) was used for cloning and sequencing in *E. coli*. Plasmid pGEM-T-easy (Promega) was used to clone polymerase chain reaction (PCR) products.

2.2. Materials

Carboxy-fluorescein-diacetate (cFDA), its succinimidylester (cFDA-SE) and 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) were from Molecular Probes Europe (the Netherlands).

2.3. Fluorescence labeling and ratio imaging microscopy

Labeling of *P. chrysogenum* mycelia with fluorescent dyes cFDA, cFDA-SE and BCECF-AM were essentially as described [27]. Mycelia of 1 ml of culture were collected by centrifugation and washed with 50 mM potassium citrate phosphate (pH 4) and subsequently incubated for 30–60 min in the same buffer supplemented with 10 μM cFDA at 24°C. For loading with cFDASE (10 μM) or BCECF-AM (10 μM), 50 mM potassium phosphate (pH 7) was used and incubation was at 35°C. After incubation, the mycelia were washed and examined by fluorescence ratio imaging microscopy [27].

2.4. Subcellular expression of the enhanced yellow fluorescent protein

To express the enhanced yellow fluorescent protein (eYFP) in the cytosol and microbody, two different constructs were made. A standard PCR reaction was performed on plasmid Kz-coxIV-eYFP [28] (kindly provided by Dr. Tsien of the University of California at San Diego) with the primer set 5′-CCCGGATCCATGGTGAGCAAGGGCGAG-3′ (forward) and 5′-GCTGTACAAGTAAAGTCGACGCGA-3′ (reverse) to yield a fragment comprising the entire eYFP coding region with the addition of a 5′ *Bam*HI site and a 3′ *Sal*I site for cloning. The primers 5′-C C C G GATCCATGGTGAGCAAGGGCGAG - 3′

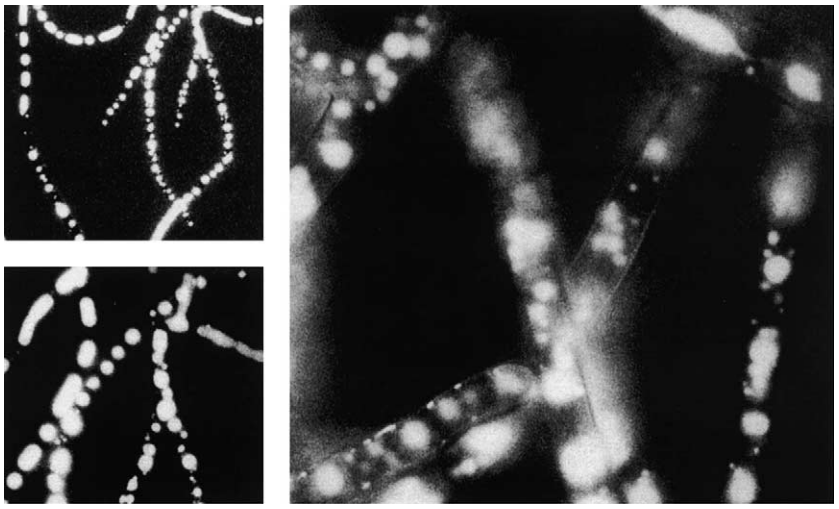

Fig. 1. Compartmentalization of carboxyfluorescein derivatives in *Penicillium chrysogenum* hyphae. *P. chrysogenum* Wisconsin 54-1255 was loaded with BCECF-AM. BCECF fluorescence was detected by fluorescence microscopy. The fluorophore partitioned in organelles of variable size. The large organelles correspond to vacuoles.

(forward) and 5′-GACGAGCTGTACAAGTCGA-AGCTGTAAGTCGACGGG-3′ (reverse) [29] (gift of Dr. K.N. Faber, Eukaryotic Microbiology, University of Groningen) were used to obtain the eYFP coding region C-terminally extended with the amino acids SKL, again with the addition of a 5′ *Bam*HI site and a 3′ *Sal*I site for cloning. Both fragments were cloned in a *P. chrysogenum* expression vector (pGBRH2, kindly provided by DSM Anti Infectives, Delft, the Netherlands) behind the *P. chrysogenum pcbC* promoter and in front of the *penDE* terminator yielding pPC-cyt-eYFP and pPC-mb-eYFP, respectively. A standard co-transformation of strains Wisconsin 54-1255 and DS04825 with pPC-cyt-eYFP or pPC-mb-eYFP with the *A. nidulans* acetamidase gene (*amdS*) gene under control of the *gpdA* promoter was conducted as described by Bull et al. [30], except that protoplasts were prepared from mycelium grown for 36 h. After transformation, colonies able to grow strongly on acetamide were selected and screened using a fluorescence microscope for the expression of eYFP.

2.5. Fluorescence measurements

eYFP fluorescence was measured using a Perkin Elmer LS-50 spectrofluorimeter, using excitation and emission wavelengths of 514 and 527 nm, respectively. The slit widths were set at 5 nm. Samples of 200 μl mycelia of the appropriate strain, grown for 2 days in penicillin production medium, were transferred to a cuvette containing a 1.6 ml of the following buffer: 25 mM KCl, 20 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 25 mM MES, MOPS, HEPES or Bicine [28] depending on the pH used. Fluorescence was monitored in time. Dissipation of the proton motive force of the cells was achieved by the addition of the protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) at a final concentration of 100 μM. The fluorescence was calibrated in the pH range of 6.5–7.5 using steps of 0.1 pH unit.

3. Results

3.1. Fluorescent labeling of P. chrysogenum organelles

Incubation of *P. chrysogenum* Wisconsin 54-1255 cells in buffer containing the fluorescent probes carboxy-fluorescein-diacetate succinimidylester (cFDA-SE) and 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), readily resulted in loading of cells with these fluorescent dyes. Even fluorescent labeling of the cytosol was obtained with the carboxyfluorescein ester cFSE, which indicated a pH of about 7 (data not shown). Loading of the cells with BCECF resulted in compartmentalization of this dye into large organ-

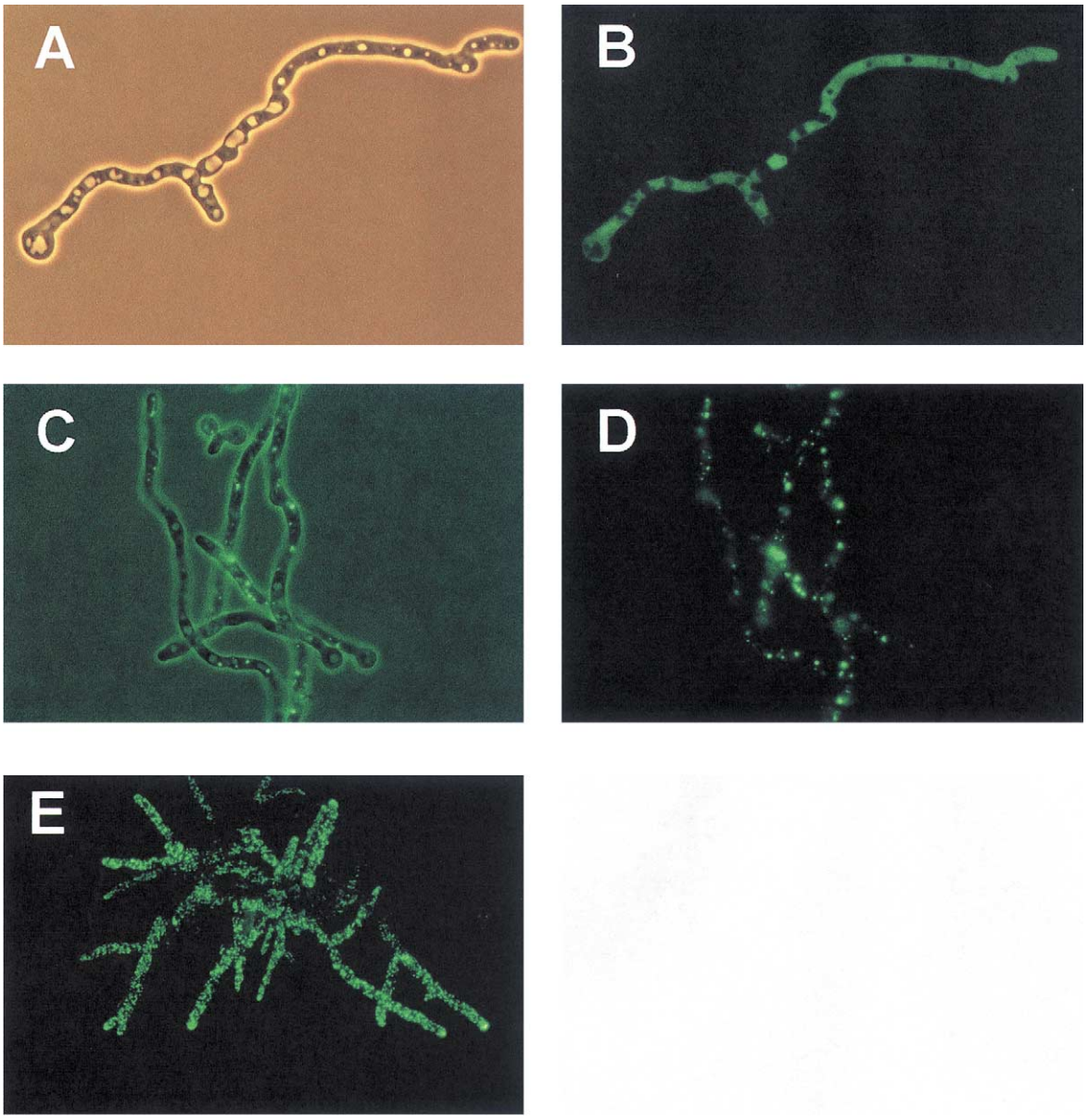

Fig. 2. Mycelia of *Penicillium chrysogenum* DS04825 transformed with eYFP. (A) Bright-field recording of a highly vacuolated hyphael cell of strain DS04825 transformed with eYFP without target sequence. (B) Fluorescent recording of same cell with fluorescent eYFP present only in the cytosol. (C) Strain DS04825 transformed with eYFP-SKL. Fluorescent recording was combined with bright field to visualize the contours of the cell. Targeting is exclusively to the microbodies. (D) Same cells as depicted in C but showing the fluorescent signal only. All mycelia were grown on lactose as the sole C-source. (E) Fluorescent recording of strain DS04825 transformed with eYFP-SKL and grown on oleate as the sole carbon source.

elles, most likely the vacuoles (Fig. 1). Also, a number of smaller organelles was labeled. Fluorescence ratio imaging spectrometry, however, showed an internal pH of the labeled organelles between 6 and 7, and this pH is not significantly different from that of the cytosol. Cells which produce high amounts of penicillin have a high content of microbodies [6]. Both the low and high penicillin yielding *P. chrysogenum* Wisconsin 54-1255 and DS04825 strain were labeled with BCECF. Fluorescence imaging of these cells showed no major difference in the abundance, size and pH of the labeled organelles (data not shown). Therefore, BCECF appears not to label the microbodies.

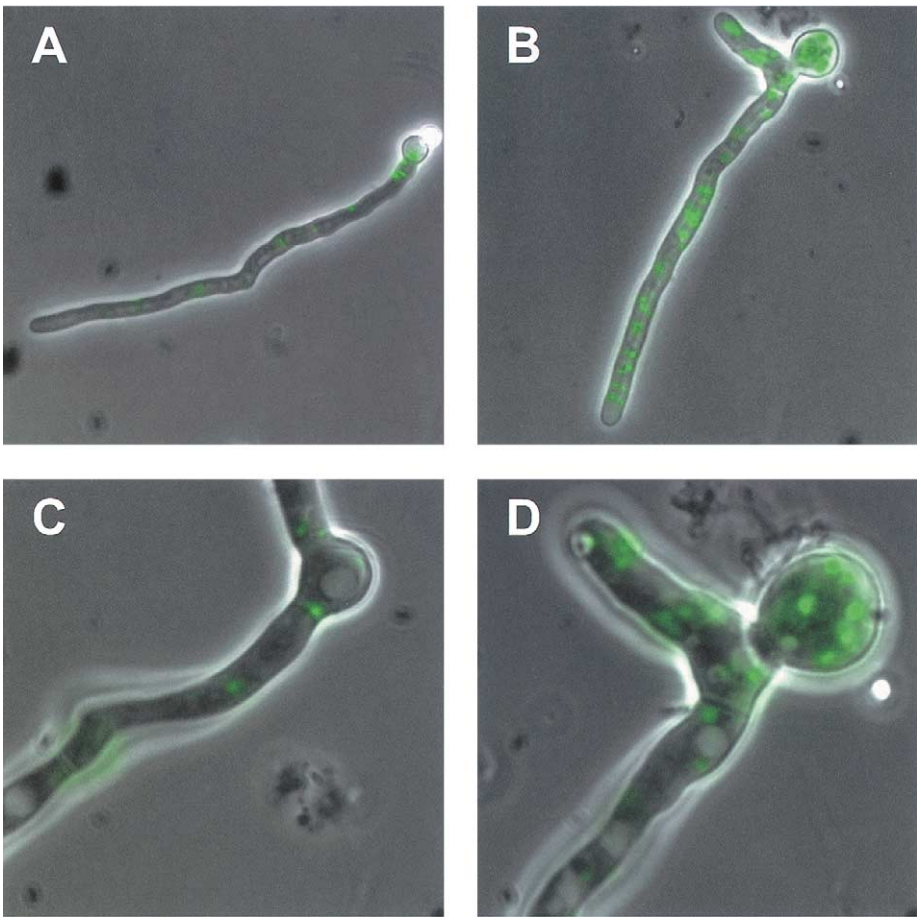

Fig. 3. Fluorescent images of germinating spores of *Penicillium chrysogenum* strain Wisconsin 54-1255 (A,C) and DS04825 (B,D) transformed with eYFP-SKL.

3.2. Subcellular targeting of green fluorescent protein derivatives

Variants of green fluorescent protein (GFP) have been constructed which show a pH-dependent fluorescence [31]. The enhanced yellow fluorescent protein (eYFP) contains four mutations (S65G/S72A/T203Y/H231L), and a near to neutral pK_a of 7.1 [28]. Therefore, eYFP can be used to monitor the pH of a cellular compartment when properly targeted to this specific organelle. To target eYFP to the microbodies, the gene optimized for human codon usage [28] was cloned in an expression vector in between the *pcbC* promoter and *penDE* terminator and extended at its C-terminus with the PTS1 microbody localization signal SKL. To target eYFP to the cytosol, a separate construct was made without the PTS1 signal. After co-transformation with a plasmid bearing the *amdS* gene, acetamide resistant colonies of strains Wis54-1255 and DS04825 were screened for expression of eYFP using a fluorescence microscope. eYFP targeted to the cytosol resulted in a uniform green fluorescence of the cytosol, while organelles (such as vacuoles) appeared as dark spots (Fig. 2A,B). Labeling of cells with eYFP-SKL led to a clear punctuate staining of the hyphae, indicating the presence of eYFP in the microbodies (Fig. 2C–E). Targeting was efficient as no fluorescence was detected in the cytosol. Interestingly, when the strain expressing eYFP in the microbodies was grown on oleate as the sole carbon source to induce the microbody located β-oxidation, the morphology of the microbodies changed dramatically (Fig. 2E).

When germinating spores of *P. chrysogenum* Wisconsin 54-1255, a strain with a relatively low capacity to produce penicillin, were transformed with eYFP-

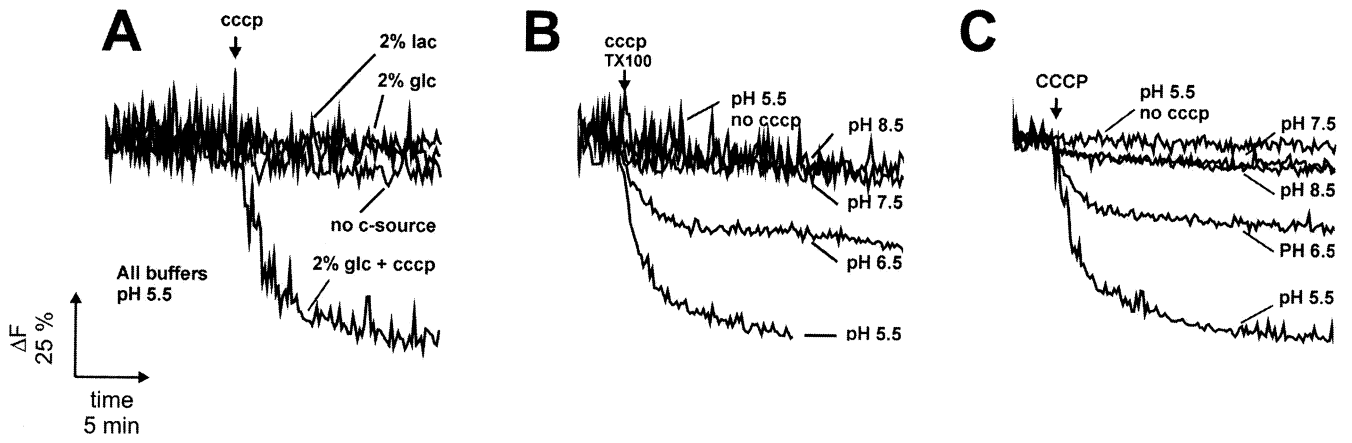

Fig. 4. pH-Dependence of the eYFP fluorescence in *Penicillium chrysogenum* DS04825 mycelia. (A,B) eYFP expressed in the cytosol, and (C) eYFP-SKL targeted to the microbodies. Additions and extracellular pH values are indicated. See text for further explanation.

SKL, only a small number of fluorescent labeled organelles were observed (Fig. 3A,C). Germinating spores of the high penicillin yielding strain DS04825 showed a large number of fluorescent organelles (Fig. 3B,D). These data are in agreement with previous electron microscopy studies that showed a high abundance of microbodies in this strain [6], and demonstrate that eYFP fused to a PTS1 signal can be used to specifically label microbodies in *P. chrysogenum*.

3.3. The pH of the cytosol and microbodies in *P. chrysogenum*

The fluorescence of eYFP was used to determine the pH of the cytosol and the microbody lumen. Mycelium of *P. chrysogenum* strain DS04825 was incubated in a buffer of pH 5.5, and the fluorescence of cytosolic eYFP was monitored in time. Since eYFP irreversibly denatures at acidic pH values, buffers with pH values below 5.5 could not be used. Irrespective the presence of glucose or lactose as energy source, the fluorescent signal remained stable for up to 25 min of incubation at pH 5.5 (Fig. 4A). The fluorescence dramatically dropped upon equilibration of the protons across the plasma membrane by the addition of the protonophore CCCP (Fig. 4A) or the ionophore nigericin (data not shown). The fluorescence was recovered to the original level when the extracellular pH was elevated at a pH slightly above 7.0 (Fig. 4B). The CCCP-induced drop in fluorescence decreased with increasing extracellular pH (Fig. 4B), allowing the calibration of the eYFP fluorescence as a function of pH. At pH values above 7.5, the eYFP fluorescence became insensitive and therefore it was not possible to accurately determine the absolute value of the cytosolic pH. The measurements, however, indicate that the pH of the cytosol is around pH 7.0–7.2.

To determine the pH of the microbody lumen, cells were transformed with eYFP-SKL. Since microbodies of the methylotrophic yeast *Hansenula polymorpha* have been reported to be acidic [21,22], eYFP with a pK_a of 7.1 should be able to record the pH in the microbody more accurately than the pH of the cytosol. However, when mycelia were incubated in a medium of pH 5.5, a stable fluorescent signal was obtained that rapidly decreased to lower values upon the addition of CCCP (Fig. 4C) or nigericin (data not shown). This implies that the pH in the microbody lumen must be significantly higher than pH 5.5. Calibration of the signal indicated a microbody luminal pH in penicillin-producing mycelia of around 7.5 (Fig. 4C). This is in the same range as the cytosolic pH. Measurements with cells induced for β-oxidation indicated a similar pH of the microbody lumen (data not shown).

4. Discussion

Compartmentalization of the penicillin biosynthetic pathway imposes intriguing questions about the role of organelles in the optimization of metabol-

ic fluxes. In particular, microbodies appear important for penicillin biosynthesis as they contain the enzymes that catalyze the final steps in penicillin production. Moreover, strains with an increased penicillin production show increasing numbers of microbodies [6]. Here, we have investigated the pH of the microbodies of *Penicillium chrysogenum* with fluorescent probes. Unfortunately, pH sensitive fluorescent dyes such as cFDA and BCECF-AM cannot be used for this purpose. Although organelles of *P. chrysogenum* could readily be stained with these dyes, they are mostly accumulated in the vacuoles. Also small organelles were fluorescently labeled (Fig. 1) but it was not possible to identify them unambiguously as microbodies. A more directed approach was chosen in which the enhanced yellow fluorescent protein (eYFP) was fused at its C-terminus with the SKL targeting signal, allowed the specific fluorescent labeling of microbodies. The number of fluorescent punctuate structures per cell increased when a strain was used that shows an increased number of microbodies (Fig. 3) in line with previous electron microscopical observations [6]. In the absence of the SKL targeting sequence, only labeling of the cytosol was obtained. The morphologies of the microbodies were different in cells grown on oleate instead of lactose or glucose as sole carbon source (Fig. 2E). This signifies the essential role of the microbodies in primary metabolism in *P. chrysogenum* similar to what has been reported for the filamentous fungus *A. nidulans* [15,16] and yeasts [31,32]. These observations firmly demonstrate that eYFP-SKL is targeted specifically to the microbodies.

Since the eYFP fluorescence is sensitive to pH, its fluorescence can be used to estimate the pH in subcellular compartments of the cell. Based on such measurements, we conclude that the pH of the cytosol and the microbody lumen in *P. chrysogenum* is nearly similar and around pH 7.0–7.5. However, an exact estimate of the pH was not possible due to the reduced sensitivity of eYFP when expressed in the cell. eYFP was reported to exhibit a pK_a of 7.1 [16], and thus should be suitable for measurements of pH values up to pH 8.0. However, under the conditions employed, the fluorescence was found to saturate at pH values above 7.5 thereby limiting the effective measuring range to pH values between pH 5.5 and 7.5. Although we have used an SKL-fused derivative of the enhanced green fluorescent protein (i.e., pK_a of 6.15 [33]), the pH dependence of its fluorescence of this protein made it even less suitable for quantification of the microbody pH in this filamentous fungus.The recent availability of a new variant of eYFP with a pK_a of 8.0 may extent the measuring range [34]. Nevertheless, the data unequivocally demonstrate that the microbody pH is not acidic and rather neutral or slightly basic. This pH is in the same range as the alkaline pH optima reported for the *P. chrysogenum* microbody enzymes acyltransferase [3,4] and acyl-CoA ligase (International patent WO97/02349). Since these enzymes are almost completely inactive at pH values of 6.0 or below, an acidic pH value as previously reported for the peroxisomal lumen in yeast [21,22] would not support enzyme activity. Notably, a recent report on peroxisomes of human fibroblasts suggests an alkaline lumen, in the range of pH 8.5–9.0 [23,35]. In that study, a fluorescent dye was chemically linked to a human peroxisomal PTS1 targeting signal that was capable to penetrate the fibroblast cells. Unfortunately, this technique could not be used with *P. chrysogenum* as the fluorescently labeled peptide was found to poorly penetrate the cell envelope while it was readily degraded (T. van der Lende, unpublished results). Since the fluorescence of eYFP as a pH-indicator can only be used to pH 7.5, we cannot exclude that also in *P. chrysogenum*, the microbody luminal pH is more alkaline. However, even though the measurable pH difference with the cytoplasm is not large, the data strongly suggest that the microbody membrane impermeable for protons in line with previous observations on microbodies in human fibroblasts [24].

Taken together, the observation that the pH of the lumen of the microbodies of *P. chrysogenum* is in the range of pH 7.0–7.5, is compatible with the pH optima of the enzymes involved in penicillin biosynthesis that are localized in this organelle.

Note added to proof

In a recent report (Jankowski, A., Kim, J.H., Collins, R.F., Daneman, R., Walton, P., Grinstein, S., J. Biol. Chem. 276: 48748-48753, 2001) a pH-sensitive mutant of GFP (pHluorin-SKL) was used to

measure the pH of intact peroxisomes in CHO cells. A luminal pH between 6.9 and 7.1 was recorded which resembles the cytosolic pH. The same study suggests that the peroxisomal membrane is highly permeable to protons.

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