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A ^{31}P NMR study of the internal pH of yeast peroxisomes

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Abstract. The internal pH of peroxisomes in the yeasts *Hansenula polymorpha*, *Candida utilis* and *Trichosporon cutaneum* X4 was estimated by ^{31}P nuclear magnetic resonance (NMR) spectroscopy. ^{31}P NMR spectra of suspensions of intact cells of these yeasts, grown under conditions of extensive peroxisomal proliferation, displayed two prominent P_i -peaks at different chemical shift positions. In control cells grown on glucose, which contain very few peroxisomes, only a single peak was observed. This latter peak, which was detected under all growth conditions, was assigned to cytosolic P_i at pH 7.1. The additional peak present in spectra of peroxisome-containing cells, reflected P_i at a considerably lower pH of approximately 5.8–6.0. Experiments with the protonophore carbonyl cyanide m-chlorophenylhydrazon (CCCP) and the ionophores valinomycin and nigericin revealed that separation of the two P_i -peaks was caused by a pH-gradient across a membrane separating the two pools. Experiments with chloroquine confirmed the acidic nature of one of these pools. In a number of transfer experiments with the yeast *H. polymorpha* it was shown that the relative intensity of the P_i -signal at the low pH-position was correlated to the peroxisomal volume fraction. These results strongly suggest that this peak has to be assigned to P_i in peroxisomes, which therefore are acidic in nature. The presence of peroxisome-associated P_i was confirmed cytochemically.

Key words: ^{31}P NMR – pH measurement – Peroxisomes – Yeast, *Hansenula polymorpha* – *Candida utilis* – *Trichosporon cutaneum*

In yeasts proliferation of peroxisomes is largely prescribed by the growth environment and due to the fact that these organelles harbour essential enzymic functions required for the metabolism of the carbon and/or nitrogen source present in the growth medium (Veenhuis and Harder 1987). In the course of our studies aimed at elucidating the physiological function and biogenesis of these organelles, we have now applied in vivo ^{31}P nuclear magnetic resonance (NMR) to simultaneously determine cytosolic and internal peroxisomal pH. This method, which relies on the pH-dependency of the chemical shifts of titratable intracellular phosphates, has been widely used to measure intracellular

pH in micro-organisms, including yeasts (Nicolay et al. 1982; Hofer et al. 1985). Recently, it has been demonstrated that ^{31}P NMR enables the simultaneous measurement of cytosolic and vacuolar pH from the (considerably different) chemical shifts of the ^{31}P NMR peaks originating from cytosolic and vacuolar P_i , respectively, in yeast grown to stationary phase on glucose (Nicolay et al. 1982, 1983). Separate peaks for the P_i -pools in these two intracellular compartments can be seen in ^{31}P spectra of these cells as a consequence of the fact that: (i) a considerable fraction of the cell volume is occupied by the vacuole; (ii) its P_i concentration is relatively high; (iii) a substantial pH gradient (0.8–1.5 units) exists across the vacuolar membrane. In the present study we investigated whether simultaneous measurement of cytosolic and peroxisomal pH by ^{31}P NMR spectroscopy was possible in yeasts, grown under conditions where peroxisomes constitute a considerable fraction of the cytoplasmic volume. In this paper evidence is presented indicating that peroxisomal P_i is indeed observed at a chemical shift position reflecting a relatively low intraperoxisomal pH (e.g. approximately pH 5.8–6.0 in methanol-grown *Hansenula polymorpha*).

Materials and methods

Organisms and growth conditions

Hansenula polymorpha de Morais et Maya CBS 4732, *Candida utilis* NCYC 321 and *Trichosporon cutaneum* X4 (Veenhuis et al. 1986) were grown in shake flasks at 37°C, 30°C and 30°C, respectively, in the growth medium of Veenhuis et al. (1986) containing 0.25% (w/v) glucose as the carbon source and 0.25% (w/v) $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source, except that for *H. polymorpha* and *C. utilis* only biotin and thiamin were used as vitamins. In addition *H. polymorpha* was grown in 0.5% (v/v) methanol medium, *C. utilis* in 0.5% (v/v) ethanol in the presence of 0.25% (w/v) ethylamine as the nitrogen source and *T. cutaneum* in 0.3% (w/v) ethylamine or 0.25% (w/v) uric acid media, respectively, as the combined carbon and nitrogen source. Cells from batch cultures were harvested from the late exponential growth phase.

For transfer of cells from methanol to ethanol- or glucose-containing media, *H. polymorpha* was cultivated in a continuous culture at a dilution rate of 0.06 h^{-1} in the medium described by Douma et al. (1985) which contained 0.5% (w/v) methanol as the carbon source.

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Abbreviations. CCCP, Carbonyl cyanide m-chlorophenylhydrazon; DCCD, N,N'-dicyclohexylcarbodiimide

Table 1

Volume fraction of peroxisomes and vacuoles in *Hansenula polymorpha*, *Candida utilis* and *Trichosporon cutaneum* X4 in relation to different growth conditions. The cells were harvested from the late exponential growth phase

Organism	Growth condition	Volume fraction of peroxisomes	Volume fraction of vacuoles
<i>H. polymorpha</i>	glucose/(NH ₄) ₂ SO ₄	0.1	6.2
	methanol/(NH ₄) ₂ SO ₄	18.9	6.4
	methanol/(NH ₄) ₂ SO ₄ (chemostat; D-0.06 h ⁻¹)	41.1	5.3
<i>T. cutaneum</i> X4	glucose/(NH ₄) ₂ SO ₄	0.2	4.2
	uric acid (C + N-source)	10.1	2.1
	ethylamine (C + N-source)	8.9	2.9
<i>C. utilis</i>	glucose/(NH ₄) ₂ SO ₄	0.2	10.2
	ethanol/ethylamine	7.8	8.9

Volume fractions are expressed as percentage of the cytoplasmic volume

Fig. 1. Orthophosphate region of ³¹P NMR spectra of aerobic suspensions of *Candida utilis* (A, B) and *Hansenula polymorpha* (C, D). Cells were grown on glucose/(NH₄)₂SO₄ (A, C), ethanol/ethylamine (B) or on methanol (D). Each spectrum represents the time-average of 2,000 scans acquired during 6.8 min. Assignments: SP sugar phosphates; P_{i,c} cytoplasmic P_i; P_{i,p} P_i presumably from peroxisomes; CW cell wall phosphomannan

Transfer experiments

For the induction of peroxisomes, cells of *H. polymorpha* and *C. utilis* were transferred to media containing methanol or ethanol/ethylamine, respectively, after extensive pre-cultivation on glucose as described previously (Veenhuis et al. 1979). In order to study the disappearance of peroxisomes, methanol-limited cells of *H. polymorpha* were diluted 6-fold in a fresh mineral medium containing 1% (v/v) ethanol or 1% (w/v) glucose and incubated in a shake flask at 37°C. Samples were taken at regular time intervals.

³¹P nuclear magnetic resonance

³¹P NMR spectra were obtained at 145.8 MHz as described previously (Nicolay et al. 1982, 1983). Usually, 500–2000 scans were averaged for a total time of 1.7–6.8 min. All experiments were carried out with washed cell suspensions which were kept at 25 ± 1°C. Aerobic conditions were maintained by sparging oxygen through the suspension as detailed before (Nicolay et al. 1983). The cell pellet volume

was usually 30–40% of the total sample volume. Under these conditions the cells were properly energized as was indicated by high levels of ATP. Also their substructure was not altered compared to 'normal' cells, as was indicated by electron microscopy of the samples after the NMR-experiments.

In a number of experiments the protonophore CCCP was added to a final concentration of 2 mM; final concentrations of the ionophores valinomycin and nigericin were 2 µM and 1 µM, respectively. Chloroquine was added to a final concentration of 40 µM.

Intracellular pH

The pH of the cytosol and peroxisomes was determined from the chemical shifts of P_i in the specific compartments according to procedures described elsewhere (Nicolay et al. 1982, 1983).

Formation of protoplasts

Protoplasts from *H. polymorpha* were prepared as described by Douma et al. (1985), from *C. utilis* as described by Zwart et al. (1983) and from *T. cutaneum* X4 as described by Veenhuis et al. (1986).

Cytochemistry

Protoplasts of *H. polymorpha*, *C. utilis* and *T. cutaneum* X4 were fixed in either 3% formaldehyde in 0.1 M cacodylate buffer pH 7.2 containing 1 M sorbitol for 30 min at 0°C or in 6% glutaraldehyde in 0.1 M cacodylate buffer for 60 min at 0°C. Fixed samples were incubated in 50 mM Bis-TrisPropane buffer pH 8.5 containing 2 mM MgCl₂, 2 mM CeCl₃ and 4 mM ATP for 2 h at 30°C. In the case of formaldehyde-fixed samples, 1 M sorbitol was added to the incubation mixture as an osmotic stabilizer. In addition incubations were performed (i) without the substrate ATP, (ii) in the presence of 10 µM DCCD as an inhibitor of ATPase activity and (iii) using 4 mM glycerol 2-phosphate instead of ATP.

After incubation, the formaldehyde-fixed samples were postfixed with 6% glutaraldehyde as described above. All samples were washed in 0.1 M cacodylate buffer pH 6.0 before postfixation in OsO₄/K₂Cr₂O₇ mixtures as described previously (Veenhuis et al. 1980).

Fig. 2. Electron micrographs showing the overall morphology of *Hansenula polymorpha* (A methanol-limited, $D = 0.06 \text{ h}^{-1}$), and *Trichosporon cutaneum* X4 (B uric acid). Abbreviations: N nucleus; M mitochondrion; P peroxisome; V vacuole. The marker represents 0.5 μm

Fig. 3. Effect of chloroquine on the orthophosphate region of ³¹P NMR spectra of aerobic suspensions of methanol-grown *Hansenula polymorpha*; A spectrum (500 scans) taken before chloroquine addition; B spectrum (500 scans) taken immediately after addition of 40 μM chloroquine at an external pH 8.5. Abbreviations as in Fig. 1

Electron microscopy

Whole cells were fixed with 1.5% KMnO_4 for 20 min at room temperature, dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300. The volume fraction of different cell organelles was determined from thin sections by the method described previously (Veenhuis et al. 1979).

Results

Figure 1 shows the orthophosphate region of 145.8 MHz ³¹P NMR spectra of aerobic suspensions of *Candida utilis* grown in glucose/ammonium sulphate medium (Fig. 1A) or in ethanol/ethylamine medium (Fig. 1B), and of *Hansenula polymorpha* grown in batch cultures on glucose/ammonium sulphate (Fig. 1C) or in a methanol-limited chemostat (Fig. 1D). The major difference between spectra obtained from glucose-grown cells (Fig. 1A and YC) as compared to those from ethanol/ethylamine-grown or methanol-limited cells (Fig. 1B and D) is the presence of a single strong inorganic phosphate (P_i) peak in the former case, while the

latter cells display two prominent P_i -peaks at different chemical shift positions. Similar differences were observed when spectra obtained from suspensions of glucose-grown *Trichosporon cutaneum* X4 were compared with those from cells grown in media containing ethylamine or urate as a carbon source (results not shown).

At the subcellular level the main morphological difference between glucose-grown cells of the various yeasts studied and those grown on the other substrates mentioned, is the prominent proliferation of peroxisomes under the latter conditions (Table 1; Fig. 2A and B). These observations suggest that the additional P_i -peak, observed in Fig. 1B and D, may originate from peroxisomal P_i . As shown previously (Nicolay et al. 1983) the prominent P_i -peak detected under all different growth conditions originates from cytosolic P_i . Separation of the two P_i -peaks, seen in cells containing many peroxisomes, suggests the existence of a pH gradient across the peroxisomal membrane which separates the cytosolic and peroxisomal P_i -pools detected by ³¹P NMR. Cytosolic pH in the different organisms, as estimated from the chemical shift position of the peak labeled $P_{i,c}$ (Fig. 1A and C), is approximately 7.1; the additional peak ($P_{i,p}$; Fig. 1B and D) reflects a considerably lower pH (approximately 5.8 in Fig. 1D).

A number of different methods were used to establish the subcellular origin of the P_i -peak in the lower pH region of Fig. 1B and D. Firstly, it could be demonstrated that a pH gradient across the membrane separating the two P_i pools is indeed responsible for the shift between their corresponding NMR peaks. Thus, dissipation of this pH gradient by the protonophore CCCP or a combination of the ionophores valinomycin and nigericin led to superposition of the two P_i -peaks. Similar observations were made upon incubation of cells with chloroquine, a drug known to accumulate in acidic compartments after being taken up in unchanged form (Lenz and Holzer 1984). As shown in Fig. 3A and B addition of chloroquine to methanol-limited cells of *H. polymorpha* at pH 8.5 immediately caused superposition of the two P_i -peaks at a position reflecting

Fig. 4A–C

Effect of changing the carbon source on the orthophosphate region of ^{31}P NMR spectra of methanol-grown *Hansenula polymorpha*. **A** control spectrum; **B** spectrum of cells harvested 1 h after changing to growth on ethanol; **C** spectrum of cells 1.5 h after changing to glucose medium. All spectra consist of 1,000 scans. Assignments as in the legend to Fig. 1

a high pH. These data indicate that the chloroquine-dependent pH equilibration between the intracellular compartments is accompanied by superposition of the two P_i -peaks.

Secondly, a number of transfer experiments were performed with *H. polymorpha* since in this organism the proliferation of peroxisomes can readily be manipulated by varying the growth conditions (Veenhuis et al. 1979, 1983). Figure 4 shows the ^{31}P NMR spectra upon a transfer of methanol-limited cells (Fig. 4A) into ethanol- (Fig. 4B) or glucose- (Fig. 4C) containing media. Clearly, the major change in these spectra is the disappearance of the P_i -peak at the low pH position of its titration curve. At the sub-cellular level these transfers are characterized by a rapid degradation of the large peroxisomes present in the cells (for details on the mechanism of peroxisomal degradation, see Veenhuis et al. 1983). Morphometrical analysis of sections of the cells indicated that the peroxisomal volume fraction had decreased approximately 12-fold and amounted to 3.4% of the cytoplasmic volume after 4 h of incubation. In controls, in which cells were transferred into media lacking the carbon source or into media supplemented with deoxyglucose [a substrate which cannot be metabolized by *H. polymorpha* and is known to prevent peroxisomal autophagy (Veenhuis et al. 1983)], the intensity of the P_i -peak at low pH remained unaltered (results not shown). A similar unmistakable correlation between the relative P_i -signal intensity and the peroxisomal volume fraction was observed in the opposite case namely when cells were transferred from media containing glucose into media that induced peroxisomal proliferation. For instance, morphometrical analysis of 3 different samples taken during exponential growth on methanol after a shift of cells from glucose, indicated that within 16 h of cultivation in the presence of methanol, the peroxisomal volume fraction had gradually increased from 0.1% to 18.9% of the cytoplasmic volume. In the mean time the vacuolar volume fraction had not significantly changed. ^{31}P NMR analysis of these samples revealed that the spectra gradually evolved from a pattern characteristic for glucose-grown cells (Fig. 1C) to that typical for methanol-grown cells (as shown in Fig. 1D).

The presence of a peroxisomal P_i -pool was confirmed cytochemically in all yeast strains studied using Ce^{3+} -ions as an orthophosphate-trapping agent (Veenhuis et al. 1980). After incubation of formaldehyde-fixed spheroplasts with CeCl_3 in the presence of ATP at pH 8.5, reaction deposits were distributed throughout the peroxisomal matrix (Fig. 5A). Similar results were obtained when isolated peroxisomes were used in these experiments. Staining of the peroxisomal matrix was strictly ATP-dependent. After incubation in the absence of ATP, or under conditions where ATPase activity was inhibited (by adding DCCD or after prolonged fixation with glutaraldehyde), no peroxisomal

staining was observed. Staining was also absent when glycerol 2-phosphate instead of ATP was used as the substrate. In the latter case only the vacuoles were stained (Fig. 5B), most probably due to alkaline phosphatase activity (Veenhuis et al. 1980).

The effect of the above formaldehyde/ATP treatment on the ^{31}P NMR spectra of *C. utilis* cells is shown in Fig. 6. Clearly a drastic increase in the P_i -signal intensity occurs at the chemical shift of the P_i -peak present at the low pH position. In view of the cytochemical data, presented above, this increase in signal must be ascribed to peroxisomal P_i . These observations lend further support to the suggestion that the P_i -peak at low pH observed in untreated cells (Fig. 1B and D) originates from peroxisomal P_i .

Discussion

In the present paper we have shown that an unambiguous correlation exists between the P_i -peak at low pH, observed in ^{31}P NMR spectra of intact cells, and the peroxisomal volume fraction of these cells in all three yeast strains studied. It should be emphasized, however, that apart from cytosolic and peroxisomal P_i as demonstrated above, under certain conditions vacuolar P_i may also contribute significantly to ^{31}P NMR spectra of yeasts (Nicolay et al. 1983). When present, the NMR peak of vacuolar P_i arises at a chemical shift position similar to that of peroxisomal P_i reported here. However, vacuolar P_i has only been observed in ^{31}P NMR spectra of yeast cells grown into stationary phase on glucose. In the current study these peaks were not observed in spectra of control samples from the exponential growth phase on glucose (see Fig. 1A and C). This essentially excludes a significant contribution of vacuolar P_i to the ^{31}P NMR-spectra presented in this study.

The present finding that yeast peroxisomes possess an acidic matrix may also explain the general observation that isolated organelles, kept in the absence of ATP, are more stable at an acidic pH (generally 5.5–6.0; Goodman et al. 1984; Veenhuis et al. 1986). Preliminary biochemical experiments indicated that this low matrix pH had little effect on the activity of main peroxisomal matrix enzymes; alcohol oxidase, isocitrate lyase and malate synthase activities, measured at pH 6.0, displayed approximately 80% of the activities determined at pH 7.0, whereas catalase activity had not decreased at all.

Our results also suggest that a proton-translocating ATPase located in or on the peroxisomal membrane may be instrumental in maintaining the observed pH difference between the cytosol and the peroxisomal matrix. In this respect it is of interest to note that DCCD, a well-known inhibitor of ATPase activity, has pronounced effects on the pH-gradient across the peroxisomal membrane. Incubation

Fig. 5. Detail of spheroplasts of *Trichosporon cutaneum* X4 **A** and *Hansenula polymorpha* (inset) showing the presence of reaction products in the peroxisomal matrix after incubation of formaldehyde-fixed samples with CeCl_3 in the presence of ATP at pH 8.5. Vacuoles remained unstained. These organelles, but not the peroxisomes were stained when glycerol 2-phosphate instead of ATP was used as the substrate (**B** *Candida utilis*-ethanol/ethylamine). Abbreviations as in Fig. 2

Fig. 6 A, B. Effect of fixation with formaldehyde followed by incubation with ATP at pH 8.5 on the orthophosphate region of ^{31}P NMR spectra of ethanol/ethylamine-grown *Candida utilis*. **A** control spectrum; **B** spectrum obtained after fixation, and incubation with ATP. For conditions see Materials and methods. 1000 scans were accumulated per spectrum. For assignments see the legend to Fig. 1

of ethanol/ethylamine grown *C. utilis* with 10 μM DCCD leads to the gradual depletion of the cellular ATP-pool which is paralleled by the progressive decrease of the above pH-gradient, as evidenced by ^{31}P NMR (data not shown). These observations suggest that a proton-translocating ATPase is involved in generating an electrochemical proton-gradient across the peroxisomal membrane. Recently we have been able to establish that such an ATPase is indeed associated with the peroxisomal membrane (Douma et al. 1987). The resulting gradient may function as a driving force for several important transport processes across this membrane.

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