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Respirational activity of *Chlorella fusca* monitored by in vivo P-31 NMR

J. Sianoudis[1], A. C. Küsel[2], T. Naujokat[2], W. Offermann[3], A. Mayer[1], L. H. Grimme[2], and D. Leibfritz[3]

Fachbereich 1 – Physik[1], Fachbereich 2 – Biologie[2], Chemie[3] der Universität Bremen, D-2800 Bremen 33, Federal Republic of Germany

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Abstract. Energy metabolism during dark respiration of the green alga *Chlorella fusca* was investigated by ^{31}P NMR spectroscopy. The kinetics of the transition from anaerobic to aerobic conditions (and vice versa) was followed with a temporal resolution of 16 s. This transition is accompanied by a shift of the cytoplasmic pH from 6.8 to 7.4, while the vacuolar pH remains constant. Simultaneously, an increase in the concentration of nucleoside-triphosphates and a decrease in the concentration of cytoplasmic orthophosphate take place, as well as the formation of "mobile" polyphosphates. The concentration of ATP and P_i reach steady-state levels within 30 s. Upon the reverse transition, from aerobic to anaerobic conditions, steady-state concentrations are obtained only after 3 min.

Key words: ^{31}P NMR, respiration, polyphosphate, Chlorella, intracellular pH

Introduction

Several authors have described ^{31}P NMR spectroscopy as a prospective tool to study the mechanisms of cell metabolism (Ugurbil et al. 1979; Gadian et al. 1979; Roberts and Jardetzky 1981). Various phosphorus metabolites are observable in the NMR spectrum of living cells, if their cellular concentration is sufficiently large. In principle, it is also possible to relate the observed signals of the metabolites to their particular environment (i.e. pH, complexation etc.) and compartmentation. In spite of these attractive prospects the actual measurements reveal several practical difficulties, which hold at least for microorganisms:

– The growth conditions within the NMR sample tube are far from being ideal, since the cell densities are too high for optimal growth. If low cell concentrations are used in the tube, long accumulation times are required.

– The observability of metabolites is limited by their intracellular concentration. Therefore, the assignment of the signals is sometimes ambiguous or incomplete.

– The discrimination of signals from the same metabolite within different cellular compartments or the assignment of one metabolite to a particular compartment is still a difficult problem.

In order to overcome some of these problems, we have chosen to work with the unicellular green alga, *Chlorella fusca* (Sianoudis et al. 1984, 1985a), because

– it allows the use of synchronized cell cultures
– the cultures are able to tolerate high cell densities and nutritional deficiencies
– the system is well established for studying the processes of dark respiration and photosynthesis.

In this paper we shall focus our attention on the metabolism involved in dark respiration. In principle, all compartments of the algal cell, i.e. cytoplasm, mitochondrion, chloroplast and vacuole(s), are involved in this metabolic process: the cytoplasm as a site of pyruvate formation and structural build up, the mitochondrion as the site of ATP replenishing, the chloroplast as the site of starch degradation and, finally, the vacuole as a source and sink of phosphate compounds.

The organization of the cell implies specific distributions of the observable phosphorus metabolites over the various cellular compartments. The adenine nucleotides (ATP/ADP) are present within the chloroplast, cytoplasm and mitochondrial matrix; inorganic orthophosphate (P_i) is present in every compartment. Polyphosphates (PP) are present both in various compartments inside the cell membrane and in the periplasmic space of *Chlorella fusca* (Sianoudis

et al. 1985b), as has been shown for other microorganisms (Ostrovskii et al. 1980; Kulaev and Vagabov 1983). Phosphorylated sugar compounds (SP) are present within the chloroplast and the cytoplasm.

Under the condition of dark respiration several potential and proton gradients are formed across the inner cell membranes between the cytoplasm, and the mitochondrial matrix. Gradients between the cytoplasm and the stroma of the chloroplast are also discussed (Bennoun 1982). Since the chemical shift of the NMR signals is particularly sensitive to the ambient pH of a given metabolite, it is possible to detect those gradients across the cell membranes by NMR spectroscopy (Moon and Richards 1973). A particularly useful candidate for that purpose is inorganic orthophosphate.

Consequently, the subjects this paper deals with are the following:

i) the attempt to discriminate between different cell compartments and their content of the above mentioned metabolites, especially P_i, in the two stationary states of cellular function, namely energized and de-energized states;

ii) the assignment of signals originating from phosphoenolpyruvate (PEP) and some phosphorylated sugar compounds (SP);

iii) the kinetics of the changes of gradients and concentrations of metabolites after turning on and off mitochondrial activity.

Experimental

Material

Chlorella fusca, strain 211-15 (Algal Culture Collection, University of Göttingen, FRG) – used in all experiments – was cultivated synchronously under a light-dark regime (14:10 h) at 28 °C, illuminated with 20 kLux on the average, or under continuous illumination with 5–7 kLux at 23 °C (asynchronous culture). The cell density starting a culture did not exceed 5×10^6 cells/ml. Details of cultivation and measurements of cell number and cell size are described elsewhere (Tantawy and Grimme 1982). For the NMR experiments the cultures were harvested, centrifuged and washed twice with phosphate-free (0.05 *M* HEPES/0.002 *M* EDTA) buffer and resuspended in the latter medium. The desired pH was adjusted with NaOH to that of the culture at the time of harvest, approximately pH 7. The suspension was concentrated up to a cell density of approximately 10^9 cells/ml. Cell extracts of *Chlorella* were obtained by perchloric acid disintegration (Navon et al. 1979).

A sample tube of 15 mm diameter was designed *i*) for supplying the cell suspension with gas, in order to maintain either anaerobic or aerobic conditions, and *ii*) for producing convection in the suspension, in order to maintain a homogeneous cell distribution within the sensitive volume. For kinetic experiments on respiration the gas flow could be switched alternately from air to nitrogen. In order to avoid effects of field inhomogeneity due to the gas bubbles and their strong convection in the sample, portions of gas were periodically admitted to the sample tube during the time interval between successive NMR pulses. For that purpose the normal acquisition time of the order of 0.3 s was prolonged with a delay to 1.2 s. The gas flow (150–200 ml/min) into the sample was pulsed with a computer-driven magnetic valve (Ogawa et al. 1978).

The necessary accumulation time for a spectrum with an acceptable signal/noise ratio limits the temporal resolution of the kinetics of metabolic processes. Under our experimental conditions (cell density, sample volume, etc.) at least 160 scans, or about 3 min of acquisition time are required. The time resolution of the response of a cell suspension to an external perturbation (i.e. the change of O_2 concentration) can be improved in the following way: the perturbation is applied repetitively with the intervals between successive perturbations being long enough in order to allow the system to relax to its stationary state. In this case a signal can be accumulated within a particular "time window" for a sufficient number of such repetitively performed perturbations. By scanning this "time window" over the total interval between successive perturbations the kinetics of the reaction can be followed. Changes from anaerobic to aerobic conditions and vice versa were performed periodically every 450 s. The corresponding O_2 concentrations within the cell suspension, as measured with an O_2 electrode, are established within about 30 s. A time window of 16 s, proved to be suitable for measuring the kinetics of the metabolic processes.

The intracellular pH was determined with respect to the chemical shift of the ^{31}P-NMR signal from inorganic orthophosphate, calibrated in the nutrient medium (Sianoudis et al. 1984).

The ^{31}P-NMR spectra were measured on a BRUKER WH 360 spectrometer, operating at 145.78 MHz in the pulsed Fourier mode, unlocked and without broad band decoupling. The pulse repetition time was between 0.3 and 1.2 s, the flip angle was between 25° (extracts) and 80° (cells). All spectra were referenced to 85% H_3PO_4 by using a coaxial capillary tube of methylene biphosphonic acid as an external standard.

The two-dimensional contour plots of time-dependent spectra were obtained with the 2D-software (Bruker Software). A series of normalized inter-

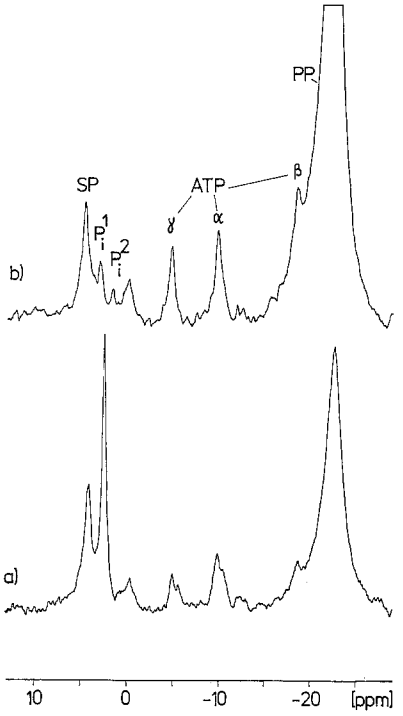

Fig. 1. ^{31}P-NMR spectrum of a cell suspension of *Chlorella fusca*, cultured under continuous light conditions; resuspended in 0.05 *M* HEPES/0.002 *M* EDTA; pH 7.0; measured in the dark under *a* anaerobic and *b* aerobic conditions. Each spectrum represents a time average of 200 scans (1.2 s/scan) (P_i^1 cytoplasmic, P_i^2 vacuolar inorganic orthophosphate)

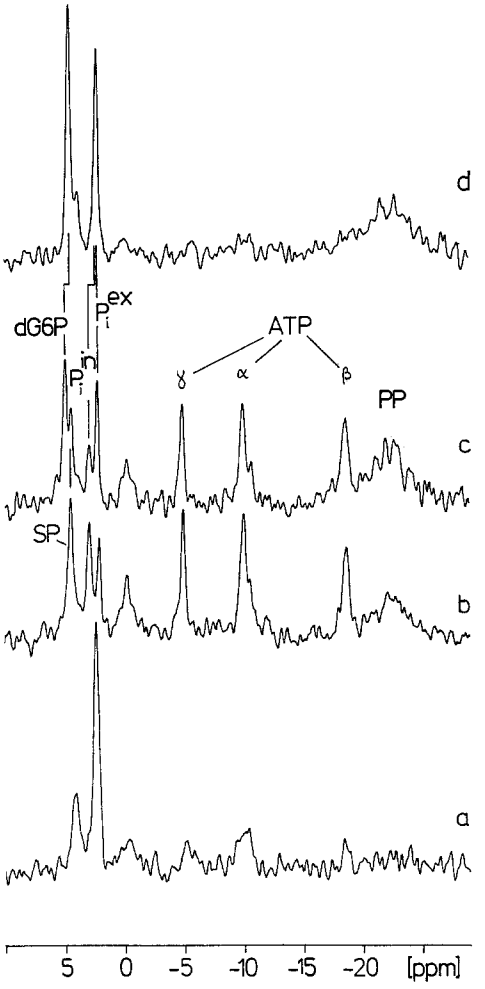

Fig. 2. ^{31}P-NMR spectrum of synchronously grown *Chlorella fusca* resuspended in the same medium as described in the legend of Fig. 1, under *a* anaerobic conditions, *b* aerobic conditions, *c* after addition of 0.5 m*M* deoxyglucose (dG), under aerobic conditions and *d* under anaerobic conditions. Each spectrum represents a sum of 160 scans (1 s/scan) (P_i^{in} intracellular, P_i^{ex} extracellular inorganic orthophosphate)

ferograms were stored in a SER-file which was transferred to a SMX-file and this was plotted as a contour plot (Leibfritz et al. 1984).

Results

The signals of the ^{31}P-NMR spectra of suspensions of *Chlorella* were identified by comparison with the spectra of lysed cells and with the aid of the data on chemical shifts reported on other cellular systems (Roberts et al. 1980; Martin et al. 1982).

Figure 1 shows the two spectra of intact cells of *Chlorella* measured under oxygen deficient (1 a) and oxygen abundant conditions (1 b). The signals (Fig. 1 b) at −4.8, −9.8 and −18.6 ppm are due to the γ-, α-, β-phosphorus of ATP, while those at −5.3 and −9.5 ppm are due to the β-, α-phosphorus of ADP respectively. Under anaerobic conditions (spectrum 1 a) the absorptions between −4.8 and −5.3 ppm, and between −9.5 and −9.8 ppm indicate the presence of both ADP and ATP. On switching to aerobic conditions (spectrum 1 b), the intensities of the ATP signals increase.

The overall intensity of ATP and ADP differs between de-energized cells and cells in the energized state (Figs. 1, 2). Such a difference has also been observed in ^{31}P NMR investigations of other systems (Stubbs et al. 1984; den Hollander et al. 1981). It may be explained either by the fact that under dark anaerobic conditions part of the ADP present in the cells is enzyme bound (Goller et al. 1982; Stitt et al. 1982) and therefore not NMR detectable and/or by the fact that a significant proportion of the adenine nucleotides is present as AMP. According to Kawada and Kanazawa (1982) the amount of AMP present in *Chlorella ellipsoidea* under dark anaerobic conditions is comparable to that of ADP and ATP.

The AMP signal occurs in the chemical shift range where the signals of other phosphomonoesters i.e. sugar phosphates (SP) also absorb (3−5 ppm, Fig. 1). The broad linewidth in the in vivo spectra

indicates either different species of phosphomonoesters and/or compartmentation. Because of this linewidth an unambiguous assignment of all phosphomonoester species, including AMP, contributing to this peak, is not possible in the in vivo spectra. A more reliable assignment is possible in spectra of cell extracts.

Comparison of the spectra in Fig. 2a, d (dark anaerobic conditions) and Fig. 2b, c (aerobic conditions) makes it clear that the possible presence of significant amounts of AMP in cells under dark anaerobic conditions cannot completely explain the difference in the signal intensity of ADP + ATP observed under aerobic and anaerobic conditions. Even if one supposed that the signal in the SP region in spectrum 2a was caused only by AMP and that no other phosphomonoesters contributed to it, there still remains a difference in the amount of adenine nucleotides observed in the spectra 2a and 2b.

The cell extracts, which were obtained by disintegration with perchloric acid, do not show the in vivo concentration of ATP and ADP because of minor hydrolysis during perchloric acid extraction.

The spectrum of a cell extract (Fig. 3) shows the β-phosphorus signal of ATP at higher field (− 20.5 ppm) than in the spectrum of an intact cell suspension (− 18.5 ppm). This indicates that in the intact cells ATP is predominantly complexed with Mg^{2+}, which was eliminated in the cell extract by the addition of EDTA (Wray et al. 1983).

Deviations in the intensity of the signal from ATP/ADP and from P_i of cells in the de-energized and energized states as seen under in vivo conditions (Figs. 1, 2) are also observed for cell extracts. The spectrum of the extract obtained from cells under anaerobic conditions (Fig. 3a) shows lower intensities of the ATP signal and higher intensity of P_i signal than does the spectrum of cells extracted under aerobic conditions (Fig. 3b).

The assignment of the peaks in the region of 4.0 to 4.8 ppm was made by the addition of reference compounds, i.e. glycolytic intermediates and AMP to the cell extracts. Thus the peak at 4.8 ppm in the spectrum of the extract of respiring cells (Fig. 3b) was assigned to glucose-6-phosphate (G-6-P).

The assignment of the peaks between 4.5 and 4.1 ppm (at the given pH and salt conditions) is difficult, because the differences in the chemical shifts between the phosphate groups of fructose-1,6-phosphate (F-1,6-P), fructose-6-phosphate (F-6-P), 3-phosphoglyceric acid (3-PGA) and AMP are small ($\Delta\delta \sim 0.1$ ppm). This is particularly true for the difference between AMP and the C6′ phosphate group of F-1,6-P as well as for F-6-P and 3-PGA ($\Delta\delta < 0.05$ ppm) (unpublished results).

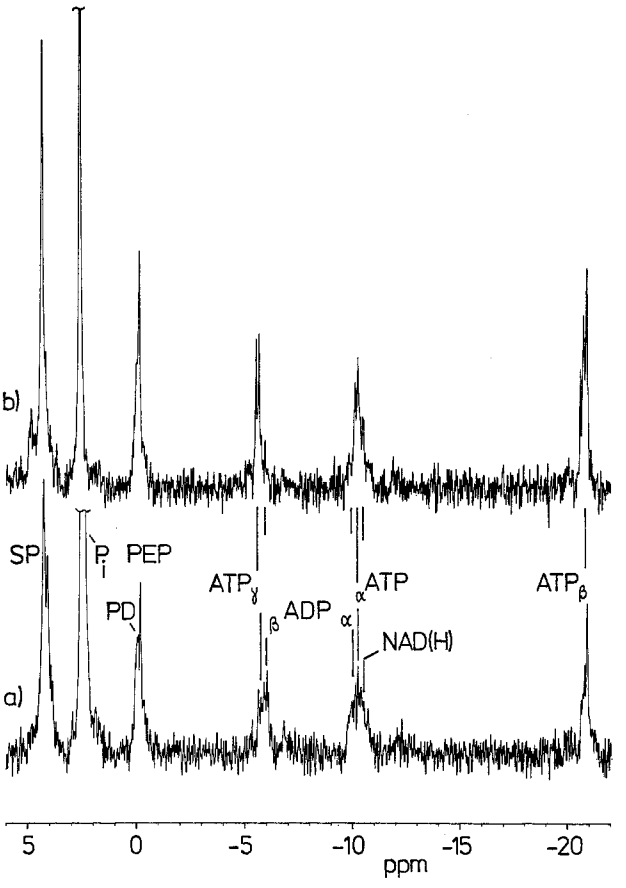

Fig. 3. ^{31}P-NMR spectra of supernatants of cell extracts of *Chlorella fusca* at pH 7.0 obtained from cells under *a* anaerobic and *b* aerobic conditions. The extracts contained 0.1 mol/l EDTA. Each spectrum is a time average of 7,280 scans (2 s/scan). The intensity of the P_i peak in spectrum *a* is twice the P_i peak intensity in spectrum *b*

Based on the results obtained by the addition of reference compounds and on titration curves we suppose that the most intense peak in both extract spectra at 4.3 ppm is due to F-6-P and/or 3-PGA. Its intensity is higher in extracts of respiring cells (Fig. 3b) than in extracts of cells under anaerobic conditions (Fig. 3a). This is in agreement with concepts on the regulation of glycolysis (Turner and Turner 1980): in respiring cells both pyruvate kinase (PK) and phosphofructokinase (PFK) show lower activity than in cells under anaerobic conditions. This should lead to an accumulation of their substrates, phosphoenolpyruvate (PEP, see below) and F-6-P respectively, as well as to higher concentrations of those glycolytic intermediates which are connected with these substrates by freely reversible reactions, namely 3-PGA and G-6-P (peak at 4.8 ppm, Fig. 3b).

To the peak at 4.1 ppm (Fig. 3a, b) both the C6′ phosphate of F-1,6-P and AMP may contribute.

Since no peak of similar intensity occurs that could be due to the C1′ phosphate group of F-1,6-P the peak at 4.1 ppm is probably mainly caused by AMP. Its total intensity is higher in the spectra of cells extracted under anaerobic conditions than in the spectra of extracts of respiring cells. This might indicate a higher concentration of AMP in cells under dark anaerobic conditions than in respiring cells. However, to some extent AMP in the extracts may result from partial hydrolysis of ATP and ADP during PCA extraction.

The signal near −23 ppm (Figs. 1, 2, and 4) originates from the core (PP_4) of mobile polyphosphate (PP) (den Hollander et al. 1981). The amount of PP within the cell depends on the particular metabolic state of the cell either during the life cycle or as a result of specific nutritional and environmental conditions (Sianoudis et al. 1984; Miyachi et al. 1964). In cells grown under continuous illumination (asynchronous culture) the amount of NMR-visible PP is higher (Fig. 1) than in synchronous cells at any stage of their growth cycle (Figs. 2 and 4).

During respiration the amount of mobile, i.e. NMR visible PP increases (Figs. 1, 2, and 4). The increase in the PP_4 peak is considerably larger than the decrease in the P_i peak, especially as other P_i-consuming processes (mainly ATP production) occur at the same time. This increase may be explained by two considerations:

i) The T_1 relaxation time of the core *P* groups of the polyphosphates is considerably shorter than of P_i: consequently, at the pulse repetition time applied, PP synthesis at the expense of P_i would lead to an unproportional increase of the PP_4 peak and decrease in P_i peak.

ii) Only a small part of the cellular PP content of *Chlorella fusca* is mobile and therefore visible in the in vivo ^{31}P NMR spectra. Thus the increase in the PP_4 peak intensity cannot, without further investigation, be attributed solely to PP synthesis but may in part be due to PP mobilization (Sianoudis et al. 1985b).

The PP are not visible in the supernatant (Fig. 3) but in the pellet of the extracts (Sianoudis et al. 1985b).

The signals of inorganic orthophosphate (P_i) appear in the region between 0.7 and 3.5 ppm (Fig. 1). Distinct peaks of P_i indicate different compartmentation of this metabolite within the cell, since the extracellular medium of the cell suspension was kept free of phosphates. The fact that both peaks arise from P_i was confirmed by the observation that they collapse in the presence of the protonophore carbonyl-cyanide-*m*-chlorophenyl-hydrazone (CCCP), (data not shown). The P_i peak near 1.6 ppm in spectrum 1b is assigned to P_i within the vacuoles (in spectrum 1a it is hidden under the large P_i peak at 2.2 ppm). The argument for this assignment is *i*) the fact that its chemical shift is consistent with an acidic environment of pH 5.7, which is characteristic for vacuoles (Smith and Raven 1979), *ii*) the observation that the chemical shift and the intensity do not vary much when the cells become energized by oxygenation. The intensity of this P_i signal depends on the age of the cell during the life cycle (Sianoudis et al. 1984). The P_i peak near 2.2 ppm shifts to 2.9 ppm upon aeration (spectrum 1b), indicating that the respective compartment becomes alkaline (from pH 6.8 to 7.4). Furthermore, the decrease in its intensity confirms the re-energetization of the cells by means of the trinucleotide synthesis. This peak is assigned to the cytoplasm by virtue of the following experiment: Deoxyglucose (dG) was added to a suspension of respiring cells. dG becomes phosphorylated to deoxy-Glucose-6-phosphate (dG6P), which is not metabolized along the glycolytic pathway during the duration of the experiment but remains in the cytoplasm (Gillies et al. 1982). Therefore its signal can be used as a marker of the cytoplasmic pH.

Figure 2c shows a spectrum of cells to which dG has been added under aerobic conditions. A large peak appeared at 5.0 ppm, which can clearly be distinguished from the SP peaks as observed in spectra 2a, b. Continued aeration led to a further increase of the intensity of this peak. It is assigned to dG6P because of its chemical shift and its intensity which is far above the maximal intensity of the SP peaks obtained from cell suspensions without added dG (spectra 1a, b and 2a, b). Upon switching to anaerobic conditions the dG6P peak shifts to 4.6 ppm (spectrum 2d). Taking into account the low pH sensitivity of the chemical shift of the dG6P peak in this pH region ($pK = 6.3$), this shift corresponds with the one of the P_i peak at 2.9/2.2 ppm respectively. Thus this P_i peak can be assigned to the cytoplasm.

A signal near −12.2 ppm is observed in some spectra, i.e. in Fig. 4 and is assigned to uridine-diphosphate glucose (UDPG). UDPG acts as a link between the primary and the polymere carbohydrate metabolism. Therefore it may accumulate during the transition from aerobic to anaerobic metabolism as a transient signal (Gibson and Shine 1983): A reduced energy supply in the cell stops the synthesis of polysaccharides and consequently leads to a temporary overshoot of UDPG.

Part of the peak at −0.2 ppm (Fig. 4) is assigned to phosphoenolpyruvate (PEP) by comparing a cell extract with one to which PEP has been successively

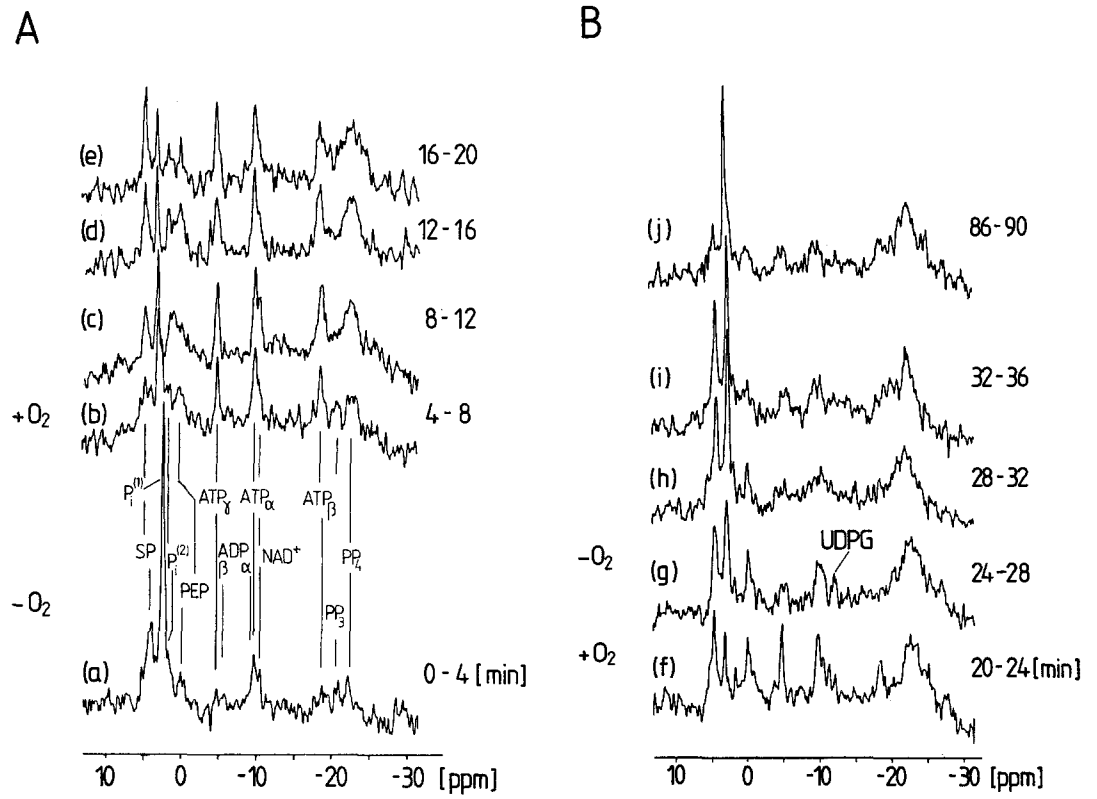

Fig. 4. **A** ^{31}P-NMR spectra of *Chlorella fusca* (0.05 *M* HEPES/0.002 *M* EDTA; ph 6.8) obtained as a function of time, when changing from anaerobic to aerobic condition in the dark. Each spectrum represents a time average of 200 scans (1.2 s/scan). **B** ^{31}P-NMR spectra of the same sample as in **A**, obtained as a function of time, when changing from aerobic to anaerobic condition in the dark. Each spectrum represents a time average of 200 scans (1.2 s/scan)

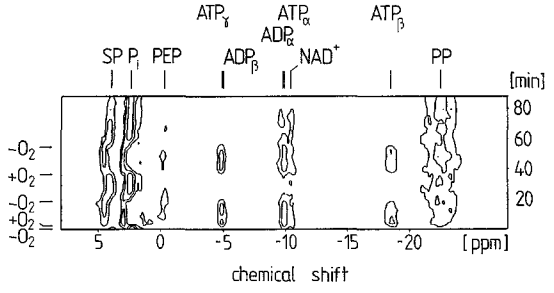

Fig. 5. A 2-dimensional representation of the ^{31}P-NMR spectra of Figs. 4A and B. The signal intensities of the metabolites are plotted as contour lines versus the time during the transitions from anaerobic to aerobic conditions and vice versa in the dark

added. Furthermore, PEP in the cell extract is decomposed at pH 8.4 within a few minutes, whereas the concentration of ATP remains unchanged under this condition. In the cell extracts the PEP signal can be clearly discriminated from the underlying signal by acidification of the extract. At pH 4.8 the PEP signal appears at − 3.15 ppm, while the other part of the original signal, which is due to phosphodiesters (PD) does not shift.

The intensity of the PEP signal increases, when the cells are transferred from anaerobic to aerobic conditions (Fig. 4). In spectra of extracts from cells disintegrated under aerobic conditions the intensity of the PEP peak is higher than in spectra of cells extracted during anaerobiosis (Fig. 3). Assuming the usual glycolytic pathway PEP is the high energy final product of glycolysis (Davies 1979). Its concentration regulates among others the phosphofructokinase (PFK) (Day and Lambers 1983; den Hollander et al. 1981). If the respiratory pathway is activated, the glycolytic activity is reduced via inhibition of PFK by the accumulation of PEP and other metabolites. The degradation of PEP itself is slowed down by competitive substrate inhibition due to the high ATP concentration from the respiratory chain.

The spectra of Fig. 4 start from a cell suspension in the dark (4 a) and describe the kinetics of the transition from anaerobic to aerobic conditions (4 b−4 f). The cytoplasmic P_i signal is shifted to lower field and its intensity decreases simultaneously with an increasing intensity of the ATP and SP signals.

The series of spectra (4 g−4 j) are from the same cell suspension in the dark and monitor the reverse

transition from aerobic to anaerobic conditions. The cytoplasmic P_i signal shifts back to higher field and its intensity increases. Simultaneously, the ATP signal loses intensity, and the intensity of the SP signal is reduced.

Figure 5 shows a 2-dimensional representation of the temporal evolution of the spectra with respirational activity. This contour plot supplies an instructive survey of the metabolic processes detected by ^{31}P NMR and shows distinctly the concentration changes of the metabolites, particularly the trinucleotides. It emphasizes the pH change upon turning on and off the respiratory activity. This can be visualized from the P_i- and SP-signal. One may see in this plot for instance that under aerobic conditions the concentration of PEP is increased, whereas under anaerobic conditions it is decreased.

The change of the cytoplasmic pH as well as the changes of the concentrations of P_i and ATP occur at a faster rate when switching from anaerobic to aerobic condition, than during the reverse transition. In order to follow the kinetics of these metabolic processes more accurately we have applied the technique of repetitive perturbation described in the experimental section.

The results of these experiments, performed with a temporal resolution of 16 s, showed that upon transition from anaerobic to aerobic conditions a steady-state level of the ATP concentration is reached within about 30 s. Alkalization of the cytoplasm is completed within 160 s. Upon the reverse transition the ATP concentration reaches a constant value only after about 3 min. The pH of the cytoplasm becomes slowly more acidic between 1 and 5 min after the cut off of the oxygen supply.

Discussion

As previous studies on various cellular systems (Ugurbil et al. 1979; Burt et al. 1979) have already demonstrated, one may learn from ^{31}P-NMR studies in particular about the role of P_i in the phosphate metabolism, regulation of PP pools and related processes. Our NMR observations on *Chlorella* show that two intracellular P_i pools can be discriminated:

– one P_i signal belongs to a neutral compartment (at the de-energized state of the cell) and is sensitive to the transition from the de-energized to the energized state of the cell, indicating an alkalization of the respective compartment. The intensity of this signal responds to changes of the metabolic state of the cells. Similar characteristics have been reported on other cellular systems (Martin et al. 1982; Nicolay et al. 1983). With the aid of dG we proved (Fig. 2), that this P_i signal originates from the cytoplasm.

– a second P_i signal corresponds to an acidic compartment. Its chemical shift and intensity remain almost the same during the transition from the de-energized to the energized state of the cells. Its intensity is influenced, however, if P_i is supplied from the exterior to the cell. Similar characteristics of this P_i signal were observed in other microorganisms, containing vacuoles (Martin et al. 1982); obviously this signal originates from the vacuole.

In the spectra presented here no further P_i peaks can be identified unambiguously. This means either that the P_i amounts present in the mitochondrion and the chloroplast of *Chlorella fusca* are below the NMR sensitivity or/and that the pH difference between these cell compartments and the cytoplasm is too small for the signals to be discriminated.

In order to resolve two P_i signals of the same intensity their chemical shift must differ by about 0.2 ppm. Furthermore whether two P_i pools of different ambient pH can be resolved does not only depend on the pH difference between these compartments, but on the absolute pH values as well (due to the sigmoidal shape of the P_i titration curve).

The ΔpH across the inner mitochondrial membrane during respiration is about 1 (Alberts et al. 1983). This would lead to a maximal Δppm of about 0.3 ppm for cytoplasmic and mitochondrial P_i in respiring cells. On the other hand the mitochondrion comprises only about 3% of the volume of a *Chlorella fusca* cell, whereas the cytoplasm amounts to about 40% (Atkinson et al. 1974).

This means that the mitochondrial P_i content would have to be very high in relation to that in the cytoplasm in order to be detectable by NMR.

It is known that the content of vacuolar P_i in *Chlorella fusca* is dependent on the cell cycle stage (Sianoudis et al. 1984). Further investigations are needed to clarify whether specific metabolic states of *Chlorella* (i.e. cell cycle stage, cells cultured under special nutritional conditions) exist during which mitochondrial P_i might be detectable.

Our data concerning the immediate influence of oxygenation of a cell suspension on the pH of the cytoplasm are in conflict with the results of Mitsumori and Ito (1984). They observed that the shift of the P_i peak, which they assigned to the cytoplasm, under oxygen abundant conditions was dependent on the time between the end of illumination (time of harvest of the culture) and the measurement. During our experiments however the pH of the cytoplasm always increased from about 6.8 to about 7.4 upon oxygenation of the cell sample, independent of the time since the last illumination.

The experiments demonstrate the functional linkage between P_i, ATP/ADP and sugar phosphates. A

quantitative analysis is not possible so far, since not all P_i consuming reactions and only the mobile species of *P*-metabolites are detectable.

The spectra of cells under aerobic and anaerobic conditions in vivo (Fig. 1 and 4) show that the amount of ATP detected in respiring cells does not equal the amount of ADP + ATP detected in cells under O_2 deficient conditions. This means that for quantitative analysis of the functional linkage of *P*-metabolites, especially the adenine nucleotides, during respiration, in vivo ^{31}P NMR spectra alone are not sufficient. They must be supplemented, i.e. by ^{31}P NMR spectra of cell extracts. Comparison of the spectra of cells extracted under aerobic and anaerobic conditions (Fig. 3) shows within the experimental error that the amount of adenine nucleotides is the same. This is in agreement with biochemical analysis (Schumann 1983).

Based on the PCA extract spectra the differences in the intensities of ADP + ATP signals in the in vivo spectra of respiring and non-respiring cells might be explained to some extent by the presence of AMP under anaerobic conditions, although this is not unambiguously proven so far. However, the presence of significant amounts of AMP cannot explain the total difference (Fig. 2a and b). This indicates that part of the adenine nucleotides present in anaerobic cells is enzyme bound, which renders them non-detectable by NMR.

Besides enzyme binding of ADP, Stubbs et al. (1984) discuss another – less likely – reason for ADP not being detectable by ^{31}P NMR in vivo, namely a rapid exchange of ADP between two cell compartments of different magnetic environment.

The polyphosphates are involved in respiratory metabolism as well, as is indicated by the formation of mobile PP in respiring cells (Figs. 1, 2, and 4). The facts that *i*) only part of the cellular PP of *Chlorella fusca* is visible in the in vivo spectra and that *ii*) there exist several fractions of PP that differ in chain length and extractability (Miyachi et al. 1964) pose special problems for the quantitative analysis of the involvement of PP in metabolic processes. It has recently been shown that the PP in *Chlorella fusca* are located both inside the cell membrane and outside in the periplasm. It is to be supposed that the PP inside the cells are not only located in the vacuoles but in other cell compartments as well (Sianoudis et al. 1985b). Which fractions of PP are involved in the formation of mobile PP during respiration remains to be investigated.

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References

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1983) Molecular Biology of the Cell. New York, London

Atkinson AW jr., John PCL, Gunning BES (1974) The Growth and division of the single mitochondrion and other organelles during the cell cycle of *Chlorella*, studied by quantitative stereology and three dimensional reconstruction. Protoplasma 81: 81–109

Bennoun P (1982) Evidence for a respiratory chain in the chloroplast. Proc Natl Acad Sci USA 79 4352-4356

Bruker Software; DISNMRP; Bruker Company; Karlsruhe/W. Germany

Burt CT, Cohen SM, Bárány M (1979) Analysis of intact tissue with ^{31}P NMR. Ann Rev Biophys Bioeng 8: 1–25

Davies DD (1979) The central role of phosphoenolpyruvate in plant metabolism. Ann Rev Plant Physiol 30: 131–238

Day DA, Lambers H (1983) The regulation of glycolysis and electron transport in roots. Physiol Plant 58: 155–160

Hollander JA den, Ugurbil K, Brown TR, Shulman RG (1981) Phosphorus-31 nuclear magnetic resonance studies of the effect of oxygen upon glycolysis in yeast. Biochemistry 20: 5871–5880

Gadian DG, Radda GK, Richards RE, Seeley PJ (1979) in "Biological Applications of Magnetic Resonance" (ed RG Shulman) Academic Press, New York, pp 463–535

Gibson DM, Shine WE (1983) Uridine diphosphate glucose breakdown is mediated by a unique enzyme activated by fructose 2,6-bisphosphate in Solanum tuberosum. Proc Natl Acad Sci USA 80: 2491–2494

Gillies RJ, Ogino T, Shulman RG, Ward DC (1982) 31-P nuclear magnetic resonance evidence for the regulation of intracellular pH by Ehrlich tumor cells. The Journal of Cell Biology 95: 24–28

Goller M, Hampp R, Ziegler H (1982) Regulation of the cytosolic adenylate ratio as determined by rapid fractionation of mesophyll protoplasts of oat. Planta 156: 255–263

Kawada E, Kanazawa T (1982) Transient changes in the energy state of adenylates and the redox states of pyridin nucleotides in *Chlorella* cells induced by environmental changes. Plant and Cell Physiol 23: 775–783

Kulaev IS, Vagabov VM (1983) Polyphosphate metabolism in microorganisms. Adv microb Physiol 24: 81–171

Leibfritz D, Offermann W, Kuhn W, Ishikawa T, Wolf KU, Becker J (1985) P-31-NMR studies on perfused rat organs (heart, diaphragm, gaster) and on human erythrocytes. In "Magnetic Resonance in Biology and Medicine (eds Govil G, Khetrapal CL, Saran A) Tata McGraw-Hill, Publ Comp, New Delhi, pp 417–427

Martin JB, Bligny R, Rebeille F, Douce R, Reguay JJ, Mathieu Y, Guern J (1982) A 31-P nuclear resonance study of intracellular pH of plant cells cultivated in liquid medium. Plant Physiol 70: 1156–1161

Miyachi S, Kanai R, Mihara S, Miyachi S, Aoki S (1964) Metabolic roles of inorganic polyphosphates in *Chlorella* cells. Biochim Biophys Acta 93: 625–634

Mitsumori F, Ito O (1984) Phosphorus-31 nuclear magnetic resonance studies of photosynthesizing *Chlorella*. FEBS 174: 248–252

Moon BL, Richards JH (1973) Determination of intracellular pH by 31-P magnetic resonance. J Biol Chem 248: 7276–7278

Navon G, Shulman RG, Yamane T, Eccleshall TR, Lam KB, Baronofsky JJ, Marmur J (1979) Phosphorus-31 nuclear magnetic resonance studies of wild type and glycolytic pathway mutants of *Saccharomyces cerevisiae*. Biochemistry 18: 4487–4499

Nicolay, K, Scheffers WA, Bruinenberg PM, Kaptein R (1983) In vivo 31-P-NMR studies on the role of the

vacuole in phosphate metabolism in yeasts. Arch Microbiol 134:270–275

Ogawa S, Shulman RG, Glynn P, Yamane T, Navon G (1978) On the measurement of pH in *Escherichia coli* ny ^{31}P nuclear magnetic resonance. Biochim Biophys Acta 502: 45–50

Ostrovskii DN, Sepetov NF, Reshetnyak VI, Siberl'dina LA (1980) Investigation of the localization of polyphosphates in cells of microorganisms by the method of high-resolation 31-P-NMR 145.78 MHz. Biokhimiya 45:517–525

Roberts JKM, Ray PM, Wade-Jardetzky N, Jardetzky O (1980) Estimation of cytoplasmic and vacuolar pH in higher plant cells by 31-P-NMR. Nature 283:870–872

Roberts JKM, Jardetzky O (1981) Monitoring of cellular metabolism by NMR. Biochim Biophys Acta 639:53–76

Schumann J (1983) Nucleotide binding to the membrane-bound chloroplast coupling factor (CF 1). Hoppe-Seyler's Zeitschr für Physiol Chem (Berling) 364:1216

Sianoudis J, Mayer A, Leibfritz D (1984) Investigation of intracellular phosphate pools of the green alga *Chlorella* using 31-P nuclear magnetic resonance. Org Magn Res 22:364–368

Sianoudis J, Naujokat T, Mayer A, Leibfritz D, Grimme LH (1985a) ^{31}P NMR studies of the phosphate metabolism of the green alga *Chlorella fusca.* in "Magnetic Resonance in Biology and Medicine" (eds Govil G, Khetrapal CL, Saran A) Tata McGraw-Hill, Publ Comp, New Delhi, pp 429–438

Sianoudis J, Küsel AC, Mayer A, Grimme LH, Leibfritz D (1985b) Distribution of Polyphosphates in cell compartments of *Chlorella fusca* by ^{31}P NMR Spectroscopy. Arch Microbiol, in press

Smith FA, Raven JA (1979) Intracellular pH and its regulation. Ann Plant Physiol 30:289–311

Stitt M, Lilley RM, Heldt HW (1982) Adenine nucleotide levels in the cytosol, chloroplasts, and mitochondria of wheat leaf protoplasts. Plant Physiol 70:971–977

Stubbs M, Freeman D, Ross BD (1984) Formation of n.m.r.-invisible ADP during renal ischaemia in rats. Biochem J 224:241–246

Tantawy MM, Grimme LH (1982) The bleaching induced by the phenylpyridazinone herbicide metflurazon in *Chlorella* is a metabolic and reversible process. Pest Biochem & Phys 18:304–313

Turner JF, Turner DH (1980) The regulation of glycolysis and the pentose phosphate pathway. In: "The biochemistry of plants", Vol. 2. Metabolism and respiration (ed Davies DD). Academic Press New York, London, Toronto, Sydney, San Francisco, pp 279–316

Ugurbil K, Shulman RG, Brown TR (1979) Biological application of magnetic resonance (ed RG Shulman). Academic Press, New York, pp 537–589

Wray V, Schiel O, Berlin J (1983) High field phosphorus-31 nuclear magnetic resonance investigation of the phosphate metabolites in cell suspension cultures of *Nicotiana tabacum.* Z Pflanzenphysiol 112:515–520