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In vivo P-31 NMR measurements of phosphate metabolism in *Platymonas subcordiformis* as related to external pH

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Abstract. The phosphate metabolism of *Platymonas subcordiformis* was investigated by 31 P-NMR spectroscopy with special attention on the effect of external pH. Glycolyzing cells and cells energized by respiration or photosynthesis gave spectra dependent upon their metabolic state. The transition from deenergized to energized states is accompanied by a shift of cytoplasmic pH from 7.1–7.4, an increase of ATP level and – in well energized cells – the appearance of a new signal tentatively assigned to phosphoarginine.

The spectra remain stable over a wide range of external pH. Cytoplasmic pH is well regulated in respiring cells for external pH in the range 5.3–12.3. The typical 0.4 units difference of internal pH in energized as compared to deenergized cells is not affected by external pH in the range 6–12. The intensity of a signal attributed to PEP is markedly increased at high external pH. pH regulation is less efficient below external pH of 6 in deenergized cells. Below pH 3.8 oxidative phosphorylation ceases. Upon raising cytoplasmic pH to 7.4 in deenergized cells polyphosphate chains start to disintegrate.

Key words: 31 P-NMR, respiration, photosynthesis, intracellular pH, polyphosphate, pH regulation

Introduction

Intracellular pH is known to play an important role in metabolic regulation (Roos and Boron 1981; Busa and Nuccitelli 1984). The extreme sensitivity of enzyme catalyzed reactions to pH changes suggests that regulation of cytoplasmic pH is fundamental to cell metabolism (Davies 1973). The response of the intracellular pH values to different conditions, especially varying external pH, reveals clues to mechanisms of pH regulation (Lane and Burris 1981; Kirst and Bisson 1982).

Several authors have used P-31 NMR spectroscopy as a tool to investigate mechanisms of cell metabolism (Gadian et al. 1979; Roberts and Jardetzky 1981). Phosphorus compounds are observable in NMR spectra of living cells at sufficiently high concentration. In some cases the observed signals give information on intracellular environment such as pH or metal ion complexation. Usually the signals are fairly broad under in vivo conditions. This is due to low motility of some metabolites, presence of paramagnetic ions, and inhomogeneities of the chemical environments of the phosphate metabolites. A further contribution to line broadening may be due to differences of magnetic susceptibilities, generating local field gradients across membranes. Signal broadening leads to lower signal to noise ratios. To avoid long accumulation times, high cell densities of more than 10^8 cells per ml are necessary. Spectroscopy on marine organisms encounters even more difficulties: higher cell densities are needed because of high electrolyte concentrations in seawater, which decreases the signal to noise ratio.

Recently green algae, such as *Chlorella fusca* (Sianoudis et al. 1985, 1986b; Mitsumori and Ito 1984), *Nitellopsis obtusa* (Mimura and Kirino 1984), and *Scenedesmus* (Ruyters et al. 1985) grown in fresh water have been investigated and show spectra which could be related to various states of glycolysis, respiration, and photosynthesis.

In order to extend P-31-NMR investigations to marine algae we chose the unicellular phytoplankton alga *Platymonas subcordiformis* (Hazen). It is a scale

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Abbreviations: PEP: Phosphoenolpyruvate; P_i: inorganic phosphate; PP_i: inorganic pyrophosphate; poly P: polyphosphates; PP-1, PP-2, PP-3: terminal, second, and third phosphate residue of polyphosphates; PP-4: core phosphate residues of polyphosphates; pH_i, pH_o: internal (cytoplasmic) and external pH; NTP/NDP: nucleotide triphosphate/-diphosphate; S/N: signal to noise ratio

bearing flagellate of the class Prasinophyceae, found in estuarine, coastal, and marine waters. As an euryhaline organism, it tolerates a wide variety of salinities. This renders it suitable for analysis of the physiological basis of ecological adaptation. It sustains adverse conditions such as high cell densities, variable osmotic stresses, variation of pH of media, and nutritional deficiencies (Kirst et al. unpublished).

In addition to the green algae mentioned above a variety of higher plant cells and tissues have been investigated using P-31-NMR with special focus on cytoplasmic and vacuolar pH (Martin et al. 1982; Roberts et al. 1980; Foyer et al. 1982; Wray et al. 1983). In cells with large vacuoles a high vacuolar phosphate signal sometimes conceals the signal of cytoplasmic phosphate, making clear determination of cytoplasmic pH more difficult (Roberts et al. 1980). The absence of a large central vacuole in *Platymonas* (Dickson and Kirst 1986) makes this algae suitable for following cytoplasmic pH from the chemical shift of inorganic phosphate (P_i) even during respiration or photosynthesis when P_i level is low.

The metabolic role of polyphosphate (polyP) in microorganisms is still under discussion (Harold 1966; Kualev and Vagabov 1983). PolyP has been investigated with P-31 NMR in several microorganisms such as *Saccharomyces cerevisiae* (Navon et al. 1979), *Cosmarium* sp. (Elgavish et al. 1980), *Endomyces magnussii, Mycobacterium smegmatis,* and various bacteria (Ostrovski et al. 1980), and with special reference to compartmentation in *Chlorella fusca* (Sianoudis et al. 1986 a).

This paper focuses on two aspects

i. the presentation and interpretation of spectra in different metabolic states: glycolysis, dark respiration and photosynthesis – and their characteristics,

ii. cytoplasmic pH and phosphate metabolism as a function of external pH.

Materials and methods

Cultures of *Platymonas subcordiformis* (Hazen) were obtained from the culture center at Göttingen (FRG) and grown in artificial sea water containing 430 m*M* NaCl in a biostat under continuous light (10 W m^{-2}) as described (Dickson and Kirst 1986). Cells assigned for measurements were transferred to culture flasks and kept in a 16 h light/8 h dark regime, for three days, bubbled with air. Algae were prepared for measurement by washing in an isotonic medium free of phosphate and free of paramagnetic ions as Mn^{2+} and centrifugation at 180 *g* for 2 min. The final pellet – 1 ml packed volume, determined by centrifugation of aliquots at 5,000 *g* for 1 min – containing $2-3 \cdot 10^8$ cells was transferred to an NMR tube (15 mm OD) and resuspended in 5 ml of artificial seawater without phosphate and microelements (e.g. manganese) buffered at pH 8 with HEPES, if constant external pH (pH_o) was desired. Reducing the content of ions in the medium led to an improvement in S/N, which was helpful when working with dilute suspensions. Experiments with an ion reduced isotonic medium (400 m*M* NaCl replaced by 650 m*M* sorbitol) showed no difference from the results reported.

Spectra were obtained on a Bruker WH 360 at 145.78 MHz in the pulse Fourier transform mode. To avoid sedimentation of the cells, the suspensions were stirred and – if desired – aerated using either the gated bubbling technique (Sianoudis et al. 1985) or continuous bubbling of the suspension with air or N_2. If the bubbles were sufficiently small which is the case for gas flow at about 30 ml/min (Ogawa et al. 1978) no adverse effects from the gas on the spectra were observed. Using the gated bubbling technique, gas flows of about 200 ml/min were used.

Illumination of cells in suspension was provided by guiding white light from a 150 W halogen bulb directly into the sample tube with a light pipe. Gated bubbling with high flow rates was usually employed to stir suspensions and provide maximal homogeneity. To supply cells with a carbon source, a gas mixture of 96% N_2 and 4% CO_2 was used.

Despite the high cell density aeration in the tube was sufficient as was shown by measurement of the oxygen tension under test conditions. Hence, anaerobic and aerobic states could be controlled experimentally. However, the extent of illumination was variable. It is difficult to assess the mutual shading effect of the cells in the tube. This certainly leads to a larger variance of cytoplasmic pH and ATP levels. The effect of illumination can be seen in suspensions bubbled with N_2/CO_2 as the change of the spectra after switching on the light.

With continuous gassing, pulse repetition times were 0.5 s, with a pulse angle of 60°. Gated gassing was used with a pulse repetition of 1 s and a pulse angle of 82°. Under these conditions spectra were accumulated within 10–20 min. Because of short longitudinal relaxation times of the polyP short pulse repetition times led to a pronounced enhancement of the polyP signal compared to the other signals. The polyP signal was less prominent with longer repetition times. Although the integrated peak areas of the different compounds do not represent their accurate relative concentration due to different relaxation times, they may be used as an estimate.

Chemical shifts were referenced against a 1 *M* solution of methylene diphosphonic acid contained

in a concentric capillary in the sample volume; each capillary was referenced against 85% H_3PO_4 yielding chemical shifts between +18.80 ppm and +19.05 ppm for different reference capillaries.

A pH calibration curve for the chemical shift of intracellular P_i was obtained by measuring shifts of P_i in a solution containing 140 m*M* KCl, 10 m*M* NaCl, 16 m*M* $MgSO_4$, 15 m*M* $CaCl_2$, 7 m*M* Na-polyP (average chain length 15 phosphates), and 20 m*M* KH_2PO_4. This ion composition resembles the intracellular concentrations of mono- and divalent cations of Platymonas as determined by Kirst (1977) and Dickson and Kirst (1986). Polyphosphate was added to take into account the complexation of metal ions to chelators, which partly reverses the pK_a lowering effect of divalent cations (Roberts et al. 1981). The concentration of phosphate residues in the core region of polyphosphate meets the corresponding intracellular concentration, as estimated from own measurements by evaluation of the integrated peak area of the polyP signal and comparison with an external standard. The resulting calibration curve is very similar to curves for cytoplasm of yeast (den Hollander et al. 1981) or the fresh water algae *Chlorella fusca* (Sianoudis et al. 1986 b).

Chemical shifts were determined with a spectral resolution of 0.03 ppm, if the signals showed the sharp peak usually seen for inorganic phosphate. This resolution allows reading of pH values from the calibration curve with a precision of ± 0.05 pH units in the steep region of the curve between pH 6.0 and pH 7.6 and ± 0.07 pH units from pH 7.6 to pH 8.0.

Titrations of external pH were done in the NMR tube by addition of small amounts of HCl or NaOH. The pH of the suspension was checked immediately before and after recording each spectrum.

Perchlorate extracts of suspensions of *Platymonas* were prepared as described (Sianoudis et al. 1985).

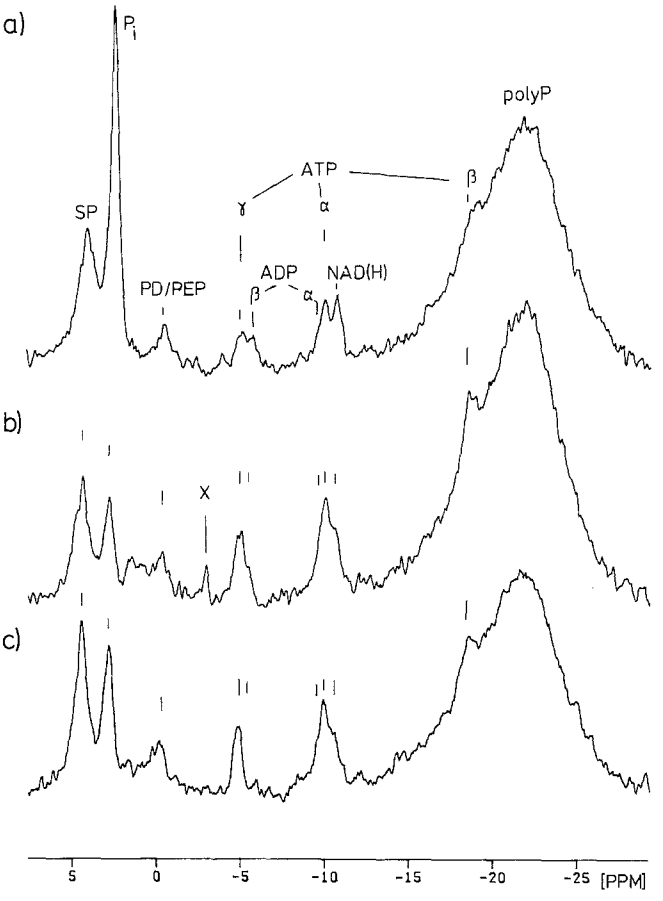

Fig. 1 a – c. 31-P-NMR spectra of a cell suspension of *Platymonas subcordiformis*, each spectrum accumulated over 608 scans using gated bubbling technique. Spectra recorded: **a** in the dark under anaerobic conditions, **b** in the dark under aerobic conditions, **c** under illumination during bubbling with N_2+CO_2

Results

Spectra of Platymonas showed different characteristics according to the various experimental conditions.

A typical spectrum of a cell suspension bubbled with N_2 in the dark is shown in Fig. 1 a. An aerated suspension – also in the dark – is given in Fig. 1 b.

Assignment of peaks is from literature data (Sianoudis et al. 1985; Navon et al. 1979) and from comparison of high resolution spectra of cell extracts with in vivo spectra.

Signals seen in the spectrum under anaerobic suspension are phosphomonoesters including sugar-phosphates (SP) at +4.1 ppm, intracellular inorganic phosphate (P_i) assigned to cytoplasm (see discussion) at +2.45 ppm, β- and α-phosphates of ADP (plus smaller amounts of other NDP) at −5.7 and −10.0 ppm, pyrophosphate diesters of NAD^+, $NADP^+$ and related compounds at −10.7 ppm, and a broad signal assigned to the nonterminal phosphate residues of inorganic polyphosphates (polyP) at −22 ppm. At about 0 ppm a fairly broad small signal is present. It contains phosphodiesters (PD) from lipids and eventually phosphoenolpyruvate (PEP).

Under aerobic conditions with the same suspension (Fig. 1 b) SP signals of the spectrum are shifted to low field at +4.4 ppm. The P_i signal is diminished and shifted to low field at +2.75 ppm. A new peak – yet unassigned – arises at −3.0 ppm; ATP signals are prominent at −4.9 ppm (γ-phosphate), −10.0 ppm (α-phosphate), and −18.7 ppm (β-phosphate). ADP is hidden under the broad ATP signals; possibly the shoulder at −5.6 ppm originates from ADP. NAD^+ and related compounds are still

visible as broad shoulder at −10.7 ppm. The polyP signal remains unchanged.

The shift of the P_i peak to low field indicates a shift of intracellular pH (pH_i) from 7.10 ± 0.05 in the anaerobic to 7.40 ± 0.05 under aerobic conditions. No signal can be ascribed to any vacuolar P_i, i.e. a P_i signal corresponding to a pH below 6 typical for vacuoles (Martin et al. 1982). Titration curves of various sugarphosphates in vitro (data not shown) indicate that the observed variation of the chemical shift of the SP signal corresponds to the pH values determined from shift of P_i. An accurate determination of pH_i using the SP signal is limited by the unknown composition of the SP.

Substitution of N_2 by a mixture of $N_2 + 4\%$ CO_2 for gassing causes no observable difference in the spectra of the anaerobic suspension. With illumination under these conditions, a spectrum similar to that of aerated suspensions in the dark was recorded (Fig. 1 c). A contour plot of spectra recorded under illumination shows the temporal evolution of the spectral features more clearly (Fig. 2): the intracellular pH shifts upwards and P_i is drastically diminished. ATP levels are elevated during photosynthesis. The signal at −0.4 ppm prominent under illumination is tentatively assigned to PEP. Each segment represents a spectrum accumulated over 5 min.

Cell suspensions were measured under different conditions at various external pH. The typical spectral features including the chemical shift difference of P_i corresponding to ΔpH_i of about 0.4 units between energized and deenergized states were stable for a wide range of pH_o. Intracellular pH − as ascertained from the chemical shift of P_i-varied only little from pH_o 5−12, i.e. over a range of 7 pH units (Fig. 3).

The two different treatments, anaerobic and aerobic, resulted in different behaviour of the cells at extreme external pH: Under anaerobic conditions, below pH_o 6, regulation of pH_i seemed to be less efficient than in more alkaline regions. pH_i decreased to about 6.4 at pH_o 4.1 compared to pH_i 7.0 at pH_o 6.5, which corresponds linearly approximated to a slope of 0.25 internal pH units per external pH unit. In the alkaline region pH increased to 7.3 at pH 12.1, corresponding to an average slope of 0.05 units pH_i/pH_o.

In the aerated suspensions pH_i increased from 7.4 at pH_o 5.3 to 7.8 at pH_o 12.3, corresponding to an average slope of about 0.06 units pH_i/pH_o as in anaerobic suspensions. Below pH_o 3.8 the internal pH decreased to 6. The disappearance of the ATP signals (Fig. 4) indicates that the energy requirements to counteract acidification exceed the energy supply by oxidative phosphorylation, so that no detectable ATP level can be maintained. This may

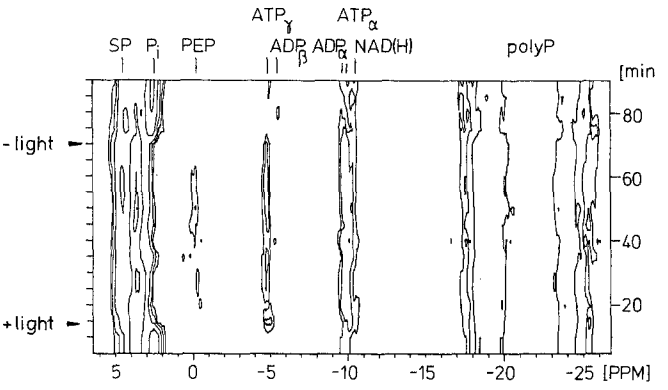

Fig. 2. 2-dimensional representation of 31-P-NMR spectra of suspension in the dark and under illumination bubbled with N_2+CO_2. Contour plot according to Offermann et al. (1987). The signal intensities of the metabolites are plotted as contour lines versus the time during transitions from the dark to illumination and vice versa. Temporal resolution corresponding to spectral accumulation time is 5 min. After 35 min a short interruption of illumination occurred

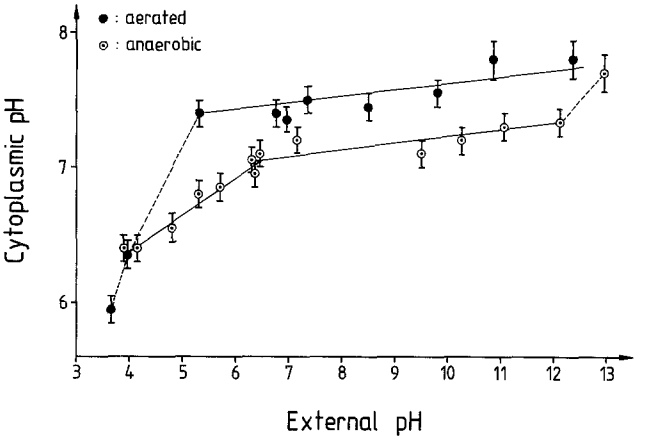

Fig. 3. Cytoplasmic pH values versus external pH in anaerobic (⊙) and aerated (●) cell suspensions, determined by 31-P-NMR. Values are taken from three different samples

be due to inhibition of oxidative phosphorylation. The spectrum looks like spectra of nonaerated cells at the same external pH (spectra not shown). Upon raising of pH_o the ATP level recovered again.

Internal pH values below 6 are difficult to estimate, because the signal of P_i approaches the SP signal, so that due to the line width of both signals it is difficult to determine the chemical shift of P_i exactly. Moreover, below pH 5.5 the chemical shift of P_i is nearly insensitive to pH.

There is a remarkable difference between aerated and not aerated cells at alkaline pH_o concerning polyP. Above pH_o 12 in anaerobic suspensions pH_i exceeded 7.3. PolyP chains started to disintegrate, as

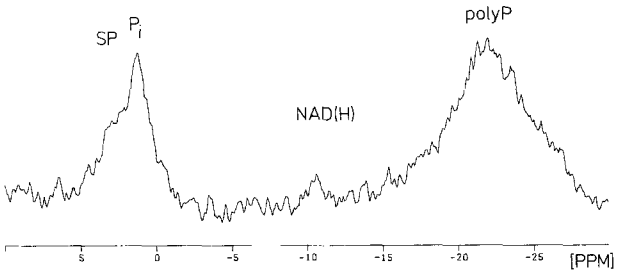

Fig. 4. Spectrum of an aerated cell suspension at pH_o 3.7, corresponding to lowest external pH in Fig. 3. The lack of an ATP signal indicates that energy requirements exceed energy supply by oxidative phosphorylation

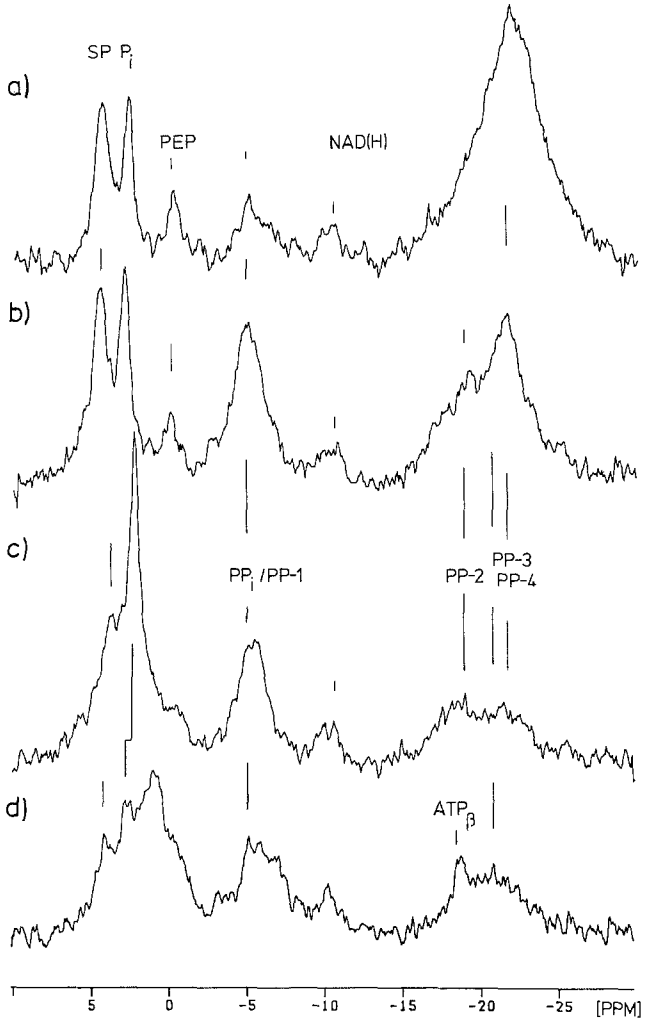

Fig. 5 a–d. Spectra showing the effect of alkalization on polyP. **a** Cell suspension under anaerobic conditions at pH_o 12.1, showing elevated PEP level and increased peak of terminal polyP (PP-1). **b** Same cells at pH_o 13.0, corresponding to the highest external pH in Fig. 3. The spectrum shows a large peak from PP_i and PP-1 and a strong shoulder from penultimate polyP (PP-2). **c** Same cells under anaerobic conditions resuspended in fresh medium at pH_o 8.0 after 20 min in alkaline medium. The small peak of polyP indicates advanced disintegration of polyP. **d** Same cells aerated at pH_o 8.0 showing elevated ATP levels and alkaline shift of cytoplasm

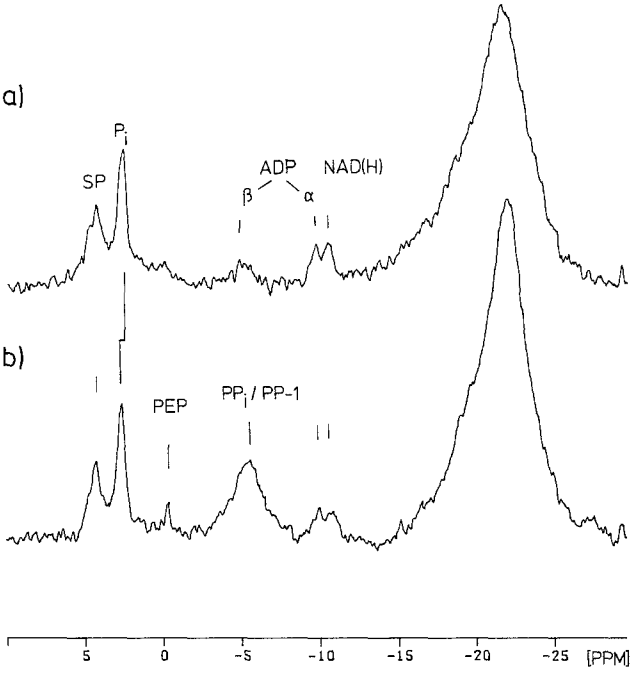

Fig. 6. a Spectrum of *Platymonas* under anaerobic conditions in medium buffered at pH_o 8. **b** The same cells after alkalization of pH_i to 7.5 by addition of 10 m*M* NH_4Cl, showing an increased peak from PP_i and PP-1

can be seen from the increase of a signal about −5 ppm which originates from inorganic pyrophosphate (PP_i) and terminal groups of polyP (PP-1) (Fig. 5 a). Growing intensity of terminal and penultimate signals of polyP relative to the core signal indicate a shortening of the average chain length.

Further alkalization of the medium to pH_o 13 enhanced the disintegration of polyP. A new signal appeared at the low field edge of the polyP core signal at −19.5 ppm (Fig. 5 b) caused by PP-2. This signal is masked by the polyP core signal in spectra showing intact polyP chains in vivo. pH_i reached 7.7 ± 0.1. About 20 min after setting the medium to pH_o 13 the algae were centrifuged and resuspended in fresh medium of pH_o 8. The change of the spectrum indicates that only a small amount of polyP remained (Fig. 5 c). The peak at −5 ppm is mainly due to PP_i. Complexation with paramagnetic ions which had been chelated to polyP before disintegration may be the reason for its large line width. The P_i level was significantly elevated, pH_i had returned to a normal value of 6.95. Investigation of the alkaline supernatant revealed that no phosphate had leaked out of the cells (spectrum not shown). The algae were still capable of oxidative phosphorylation, as concluded from the spectrum during aeration (Fig. 5 d). Typically this could be seen as an alkaliza-

tion of pH_i, increasing by 0.4 units compared to the anaerobic conditions. ATP levels were significantly elevated. The PP_i peak decreased and a new broad signal at +1 ppm appeared. After 30 min the PP_i peak had disappeared almost completely (spectrum not shown).

Disintegration of polyP could also be observed after artificial alkalization of pH_i to 7.4 by adding 10 m*M* of NH_4Cl to the cell suspension buffered at pH 8 under anaerobic conditions (Fig. 6).

Remarkably, polyP is not affected if the intracellular pH is elevated due to respiration or photosynthesis. As noted before, pH_i may reach 7.8 under these conditions. Even after alkalization of the external medium to pH 12.4, no effect on the polyP pool was observed.

Discussion

i. Phosphate metabolism at glycolysis, respiration and photosynthesis

The spectral features of *Platymonas subcordiformis* in the various metabolic states, namely anaerobic dark, anaerobic light, and aerobic dark resemble those which were observed for the unicellular green alga *Chlorella fusca* (Sianoudis et al. 1985, 1986 b), although there are some significant differences. There is an intriguing resemblance of the spectra of anaerobic light and aerobic dark conditions. This is true particularly with respect to the intracellular P_i signal and its shift upon transition from the deenergized to the energized states. Only one single intracellular P_i signal is observable in the various metabolic states of *Platymonas subcordiformis.* One would expect to deal with at least four signals of P_i pertinent to the different compartments in the cell (vacuoles, cytoplasm, mitochondria, chloroplasts). We believe the observed P_i signal originates mainly from the cytoplasm, for the following reasons.

There are no large vacuoles in *Platymonas.* Vacuolar P_i would be expected at about +1 ppm corresponding to a pH below 6, being typical for vacuoles in algae such as *Chlorella* (Sianoudis et al. 1985) and higher plants (Martin et al. 1982). As another example *Lamprothamnium,* a characean alga living in a marine like environment as *Platymonas,* has a vacuolar pH of 5 at external pH below 8 (Kirst and Bisson 1982).

Mitochondria have to be eliminated as compartment of a detectable P_i-pool because they occupy too small a volume inside the cell and would not cause a large P_i signal.

As in most microalgae the chloroplast occupies about 40% of the cell volume in *Platymonas,* which is comparable to *Chlorella fusca.* In this alga the chloroplast accounts for about 30% of cell volume. However, in *Chlorella* it was shown (Sianoudis et al. 1985, 1986 b) by comparing variations of the chemical shift of P_i with those of deoxy-glucose-6-phosphate, a compound known to accumulate in the cytoplasm, that the P_i signal originates from cytoplasm.

The presence of other P_i signals cannot be totally excluded though. P_i contents of the stroma in plastides and/or mitochondria may be masked by the fairly broad cytoplasmic P_i signal. A pH difference of about 1 unit – characteristic for the inner mitochondrial membrane during respiration (Alberts et al. 1983) – would result in a shift difference of about 0.4 ppm between cytoplasmic and mitochondrial P_i in respiring cells.

The same consideration holds true for stromatic P_i in photosynthesizing cells. In the spectra obtained under illumination such a second P_i signal cannot be detected unambiguously. The possibility that two signals of P_i coincide because of fast exchange of inorganic phosphate can be ruled out following the argument in Sianoudis et al. (1986 b). It still remains possible that the P_i signals from different compartments coincide either because of equal pH or because different ion concentrations counteract pH induced chemical shifts.

Typical changes in the spectra are connected to the transition from deenergized to energized cells.

The decrease of P_i level accompanied by increase of the ATP level is the direct response to oxidative phosphorylation respective photophosphorylation. Not all phosphate missing in the P_i peak would reappear as ATP. Phosphate pools not detectable by NMR must be taken into account as only freely rotating metabolites can be detected by in vivo NMR. Immobilization of phosphate residues by bonding to macromolecules or membranes leads to an extreme line broadening so that the NMR signal disappears into the baseline of the spectrum. Enzyme bonding is believed to be responsible for partial invisibility of ADP (Sianoudis et al. 1985).

The alkalization of the cytoplasm in energized cells is typically 0.4 to 0.6 pH units compared to deenergized cells, averaged over a large number of different samples. It resembles results of NMR measurements with *Chlorella* (Sianoudis et al. 1985, 1986 b) and *Nitellopsis obtusa* (Mimura and Kirino 1984).

The signal denoted by SP – sugarphosphates – includes different phosphomonoesters, among them hexose and triose phosphates. In perchlorate extracts the signal is split into five peaks, very likely deriving

from fructose-6-phosphate, glucose-6-phosphate, and fructose-1.6-phosphate, which are known to be present in the cytoplasm. Triosephosphates as 3-phosphoglycerate, 1,3-diphosphoglycerate, glycerine-aldehyd-3-phosphate, and dihydroxyacetonphosphate are present preferably in the chloroplast (Fig. 7).

The signals in the region between +1 and −1 ppm originate mainly from phosphodiesters. Their intensities are usually rather low and they vary considerably in different samples. The peak at −0.4 ppm is partly due to PEP (Sianoudis et al. 1985 a). This tentative assignment is supported by the fact that the signal is increased during photosynthesis (Fig. 2) and during alkalization of the external medium (Fig. 5 a, b). PEP is a precursor of organic acids which are very likely synthesized to counteract alkalization of cytoplasm (Smith and Raven 1976).

To our knowledge the signal at −3.0 ppm has not been reported before in plant cells. Its chemical shift is typical for phosphagen compounds, resembling that of phosphoarginine. PArg is known to serve as energy reserve in muscles of insects and molluscs. It has been reported in P-31 NMR spectra of the marine mollusc *Tapes watlingi* (Barrow et al. 1980) and in spectra of the ciliated protozoan *Tetrahymena* (Findly et al. 1983). In *Platymonas* it might serve in similar manner. This alga possesses a comparatively large rhizoplast, an apparatus which supports the flagella movement in a muscle like manner (Salisbury and Floyd 1978). It is speculated that this rhizoplast is energized by a metabolic pathway similar to muscle action known in lower animals. The fact that a large PArg signal was only recorded in aerated suspensions may be due to insufficient energization by illumination rather than to a metabolic difference between respiration and photosynthesis.

The assignment of ADP, ATP and NAD^+ is straightforward. The perchlorate extract spectrum (Fig. 7) shows the typical patterns of the compounds: two doublets of ADP, two doublets of γ and α phosphate of ATP, the triplet of β-phosphate of ATP, and the quadruplet of NAD^+ at the expected positions. In the in vivo spectra ATP and ADP are not distinguishable by their shifts, due to the broad signals. Presence of ADP in the energized cells may be inferred from shoulders at the ATP signals.

The broad signal at the high field end of the spectrum is due to long chain polyphosphate. Its intensity varies with different samples. The position at −22 ppm is in accordance with complexation to Mg^{2+} and Ca^{2+} in concentrations known to occur in the cytoplasma (Dickson and Kirst 1986; own measurements). The core phosphates (PP-4) are found at 22.0 ppm, while penultimate phosphates lie at −21.1 ppm (PP-3) and −19.1 ppm (PP-2). These positions are within the range of the broad core signal, so that no separate signals from the penultimate signals can be distinguished under in vivo conditions.

The large line width of the polyP core signal is characteristic of the presence of paramagnetic ions such as Mn^{2+} and Fe^{2+}, which are part of the culture medium. They are possibly complexed to the polyP chains together with Mg^{2+} and Ca^{2+}. A further con-

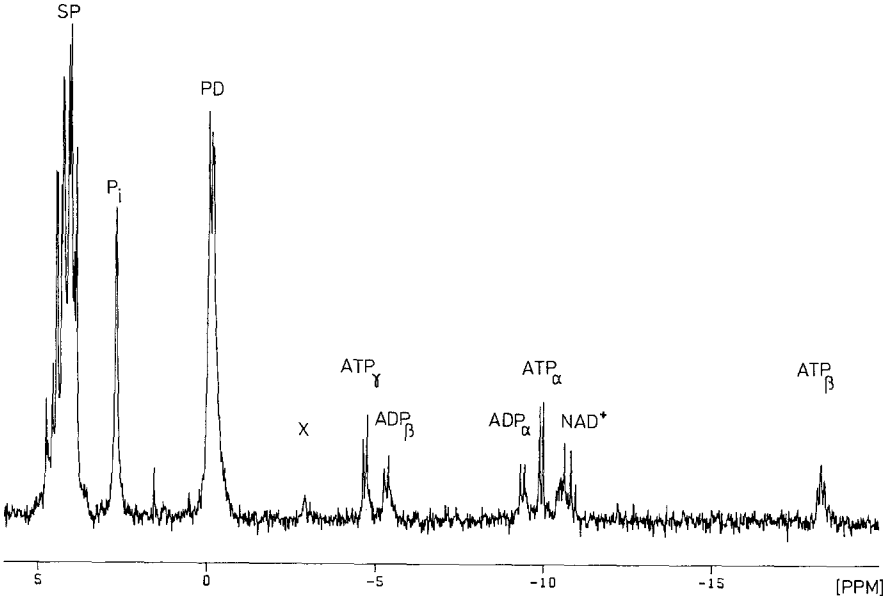

Fig. 7. Perchlorate extract of *Platymonas subcordiformis;* pH adjusted to 7.0; divalent cations partially removed by addition of EDTA in a small amount

tribution to the line width may be due to a reduced motility of the chains, which leads to a strong effect of chemical shift anisotropy.

The fact that terminal phosphates of polyP do not cause a detectable signal at −5 ppm and that the penultimate phosphates are only weak − as concluded from the symmetric shape of the core signal − can be interpreted as that either the chains are very long or the terminals are not mobile because of bonding to proteins or cell walls (Harold 1966).

Chain lengths of more than 40 residues as reported from several algae are consistent with the first interpretation (Glonek et al. 1970; Miyachi et al. 1964), though the second argument cannot be excluded.

ii. Dependence of cytoplasmic pH and phosphate metabolism on external pH

The absolute determination of pH depends on the precise reproduction of the cytoplasmic milieu in vitro for calibration. In the range of maximal slope of the calibration curve − between pH 6.0 and pH 7.6 − the difference of P_i in pure water and P_i in the presence of 1.6 *M* KCl is about 0.5 units (data from Gadian et al. 1979). Smaller amounts of divalent cations − 50 m*M* Mg^{2+} − cause the same effect.

Considering the composition of the cytoplasma of *Platymonas* containing about 200 m*M* of monovalent cations and 20 m*M* of Mg^{2+} the systematic error will be well below 0.5 pH units. The most important uncertainty is the correct amount of polyP to be added to the calibration medium. The addition of Mg^{2+} shifts the pK_a of the calibration curve to lower values, which is reversed if added polyP complexes Mg^{2+}. In the present case, the addition of polyP shifts the pK_a back to higher values to such an extent that the determined calibration curve resembles that for fresh water organisms as *Chlorella* (Sianoudis et al. 1986 b) and yeast (den Hollander et al. 1981). If − contrary to our assumption − the polyP present in *Platymonas* were not accessible to cytoplasmic Mg^{2+}, or if polyP complexed fewer cations in the cytoplasm than in vitro, there would be more free Mg^{2+} in solution. The actual pK_a would be lower and therefore pH would be overestimated in that case. Proteins and neutral organic solutes added to account for further components present in the cytoplasm have no effect on the calibration (Roberts et al. 1981). Charged organic solutes may, however, shift the curve to a certain extent.

The present calibration is supported by the fact that core polyP appears at the same frequency in the calibration medium as in vivo − at −22 ppm. Its shift is sensitive to the concentrations of Mg^{2+} (own measurements). Hence different ion compositions would show up as a different chemical shift of polyP.

Consequently, the determination of pH differences in a single sample is reliable within the resolution of the chemical shift of P (±0.03 ppm), corresponding to ±0.05 pH units. This is much more precise than other known methods to determine internal pH. The displacement of the curve originating from mismatch of the calibration medium is assumed to be below an amount corresponding to a systematic error of 0.2 pH units, which would result in too high rather than too low values, as explained above.

The fact that the spectra typical for energized and deenergized cells and the typical pH difference of 0.4 pH units between glycolyzing and respiring cells is constant over a wide range of external pH indicates a powerful regulation of cytoplasmic pH especially in the alkaline region, which is useful for *Platymonas* in their natural environment. These algae live in sea water at about pH 8. They tolerate high cell densities, where external pH may rise up to pH 11 during photosynthetic activity under illumination (Kirst, unpublished).

A possible mechanism to keep pH low is the synthesis of organic acids (Smith and Raven 1976) with PEP as precursor. Spectra of algae in alkaline medium exhibit an enhanced PEP signal (Fig. 5 a, b) which indicates the participation of this mechanism in pH regulation. PEP-level is also high in aerated cells at pH_o 12.1 (spectrum not shown) and in cells, where cytoplasm is alkalized by NH_4Cl at normal pH_o of 8 (Fig. 6 b).

While in the alkaline region pH regulation is as effective in energized as in deenergized cells, anaerobic cells exhibit a steeper decrease of pH_i with decreasing pH_o than aerobic cells in acid medium where pH_o is less than pH_i. Below pH_o 4, when energy requirements exceed energy supply by oxidative phosphorylation, pH_i of anaerobic and aerobic cells fall to the same value. Their spectra are similar. The difference between energized and deenergized cells as well as the failure of pH regulation after breakdown of oxidative phosphorylation indicates that pH regulation under acid conditions is more energy demanding.

The fresh water algae *Chlorella pyrenoidosa* and *Scenedesmus quadricauda,* regulate pH_i even at external pH of 3 (Lane and Burris 1981). The euryhaline charophyte *Lamprothamnium* regulates pH_i at pH_o 5 and regulation fails above pH_o 9 (Kirst and Bisson 1982).

In the metabolism of many microorganisms inorganic polyphosphates function both as a phosphate storage compound and as energy rich mole-

cules (Kualev and Vagabov 1983). Studying effects of unphysiological high pH_o reveals clues on compartmentation of polyP (Sianoudis et al. 1986 a).

There is a significant increase of pH_i in anaerobic cells in the alkaline region above pH_o 12, accompanied by disintegration of polyP. This also occurs on alkalization of cytoplasm by adding NH_4Cl (Fig. 6). Disintegration of polyP does not counteract alkalization of the cytoplasma. The fact that under aeration even higher cytoplasmic pH does not affect polyP indicates that the disintegration is not a chemical pH effect but rather due to a metabolic process.

In contrast to the findings of Sianoudis et al. (1986 a) with *Chlorella fusca* there is no sharpening and displacement of the signal due to decomplexation of Mg^{2+} in the alkaline region. In *Platymonas subcordiformis* therefore polyP is not situated in a periplasmic space or another site accessible to the external medium. The fact that polyP is sensitive to an alkalization of the cytoplasm together with the consistency of its chemical shift with the shift measured for polyP in a solution replicating cytoplasmic ion concentrations indicates that polyP is freely accessible from the cytoplasma.

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