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P-31 NMR saturation transfer experiments in *Chlamydomonas reinhardtii*: evidence for the NMR visibility of chloroplastidic P_i

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Abstract. ATP synthesis and consumption in respiring cells of the green alga *Chlamydomonas reinhardtii* were measured with ^{31}P in vivo NMR saturation transfer experiments to determine the intracellular compartmentation of inorganic phosphate. Most of the observed flux towards ATP synthesis was catalyzed by the coupled enzymes glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase (GAPDH/PGK). The attribution of the measured flux to these enzymes is supported by the observation, that (i) the magnetization transfer was strongly reduced by iodoacetate, an irreversible inhibitor of GAPDH and that (ii) the unidirectional flux was much greater than the net flux through the mitochondrial F_0F_1-ATPase as determined by oxygen consumption measurements. In *Chlamydomonas*, glycolysis is divided into a chloroplastidic and a cytosolic part with the enzymes GAPDH/PGK being located in the chloroplast stroma (Klein 1986). The ^{31}P-NMR signal of inorganic phosphate must, therefore, originate from the chloroplast. The life time of the magnetic label transferred to P_i by these enzymes is too short for it to be transported to the cytosol via the phosphate translocator of the chloroplast envelope. When the intracellular compartmentation of P_i was taken into consideration the calculated unidirectional ATP synthesis rate was equal to the consumption rate, indicating operation of GAPDH/PGK near equilibrium. The assignment of most of the intracellular P_i to the chloroplast is in contradiction to earlier reports, which attributed the P_i signal to the cytosol. This is of special interest for the use of the chemical shift of the P_i signal as an intracellular pH-marker in plant cells.

Key words: ^{31}P-NMR – *Chlamydomonas* – Saturation transfer – Chloroplast – Intracellular pH

Abbreviations: 3-PGA: 3-phosphoglycerate, CW: continuous wave; dG6P: 2-deoxyglucose-6-phosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; M_0: equilibrium z-magnetization; M_t: instantaneous z-magnetization after selective saturation for time t; MDP: methylene-diphosphonic acid, PDE: phosphodiester; PGK: phosphoglycerate kinase; P_i: inorganic orthophosphate; polyP: polyphosphate; T_1: longitudinal relaxation time; τ_1: longitudinal relaxation time with chemical exchange; TCA cycle: tricarboxylic acid cycle

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Introduction

^{31}P-NMR is a non-invasive method well suited to the measurement of intracellular concentrations of phosphate metabolites in the living organism. Special interest in the resonance of intracellular orthophosphate arose from the pH-dependency of its chemical shift (Moon and Richard 1973) which can be used to determine the intracellular pH. A further advantage of ^{31}P in vivo NMR is the ability to discriminate between different subcellular compartments. This aim is usually difficult to achieve with conventional analytical methods and requires the isolation of the organelles. In most cases only two intracellular P_i signals have been detected in ^{31}P in vivo NMR spectra of algae measured under aerobic conditions in the dark (Mitsumori and Ito 1984, Sianoudis et al. 1985, Kugel et al. 1987, Bental et al. 1988, Lundberg et al. 1989). These P_i signals can originate from at least four cell compartments: chloroplast, cytosol, mitochondrium or vacuole.

The P_i signal which indicates an acidic pH has been attributed to the vacuole (Waterton et al. 1983, Sianoudis et al. 1984). The assignment of the remaining extravacuolar P_i signal to a specific intracellular compartment, however, is still controversial. The intensity and chemical shift of this P_i signal were found to be dependent on the energy status of the cell. The chemical shift indicated a more alkaline pH when the experimental conditions allowed respiration or photosynthesis than when the cells were kept anaerobic in the dark (Sianoudis et al. 1987). It was suggested to attribute this signal to the chloroplast (Waterton et al. 1983, Mitsumori and Ito 1984) to explain the alkalization under illumination, an observation which is also known from isolated chloroplasts of spinach (Heldt et al. 1973, Heber and Heldt 1981).

An experimental approach to clarify the intracellular localization of P_i is to introduce a further pH-marker with known intracellular compartmentation e.g. 2-deoxyglucose-6-phosphate (dG6P). The pH indicated by its chemical shift can then be compared with the pH derived from the P_i resonance. After addition of 2-deoxyglucose to cell suspensions of the green alga *Chlorella fusca* an intracellular accumulation of dG6P was observed. The pH-values indicated by the signals of dG6P and the extravacuolar P_i showed strong coincidence under anaerobic and aerobic conditions (Sianoudis et al. 1985) and under illumination (Sianoudis et al. 1987). These results led to the conclusion that both signals originate from the same compartment. Since it is known from mammalian cells that dG6P accumulates in the cytosol (Navon et al. 1977, Gillies et al. 1982), the P_i was also attributed to the cytosol in green algae (Sianoudis et al. 1987, Lundberg et al. 1989). As no additional P_i signal was observed it was assumed that either the pH-difference between cytosol and chloroplast is too small to be resolved or that the concentration difference between both P_i pools is too large to detect both signals. Furthermore, a reduced NMR visibility of the chloroplastidic P_i was proposed, because part of the P_i could be bound to enzymes (Sianoudis et al. 1987).

However, there is some uncertainty about the intracellular compartmentation of dG6P in plant cells. It has been shown that glucose can be transported across the envelope of intact spinach chloroplasts (Schäfer et al. 1977). Also the phosphorylated species glucose-6-phosphate can be taken up by plastids isolated from *Codium fragile* (Rutter and Cobb 1983) or pea roots (Borchert et al. 1989).

To circumvent the problem of an uncertain intracellular distribution of the pH-marker substance, we used a magnetic labelling technique to investigate in vivo a specific P_i consuming enzyme reaction which can definitely be attributed to a certain cell compartment. In the green alga *Chlamydomonas reinhardtii*, glycolysis is divided into a chloroplastidic and a cytosolic part. The enzymes which catalyze the conversion of glucose to 3-phosphoglycerate (3-PGA) are mainly located in the chloroplast stroma whereas those catalyzing the subsequent reactions from 3-PGA to pyruvate are located in the cytosol (Klein 1986). The distinct subdivision of the glycolytic pathway is also reflected in the corresponding distribution of the glycolytic metabolites (Klöck and Kreuzberg 1991).

We focused on the coupled glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase (GAPDH/PGK) reaction. In *Chlamydomonas* more than 85% of the NAD-dependent GAPDH and all of the NADP-dependent GAPDH activity of whole cells was found in the isolated chloroplasts (Klein 1986). Magnetic saturation transfer experiments on *Saccharomyces cerevisiae* (Brindle and Krikler 1985, Campbell-Burk et al. 1987, Brindle 1988 b) and *Escherichia coli* (Mitsumori et al. 1988) showed that the coupled glycolytic enzymes GAPDH/PGK are operating near equilibrium and catalyze large unidirectional fluxes in either direction, which can be followed by NMR.

In the present work we show that GAPDH/PGK also operate near equilibrium in *Chlamydomonas reinhardtii*. They catalyze large unidirectional fluxes of P_i towards ATP. Because the substrates involved must be in the same compartment as the enzymes we conclude that the alkaline P_i signal in the ^{31}P in vivo NMR spectra has to be attributed to the chloroplast.

Material and methods

Cell culture and preparation of Chlamydomonas for NMR measurements

Chlamydomonas reinhardtii wild type 137c was grown photoautotrophically at 28 °C in a modified high-salt culture medium (Sueoka 1960). The concentrations of the paramagnetic ions Fe^{2+}, Mn^{2+} and Co^{2+} were reduced by more than 90%. The algae were synchronized by periodic illumination (12 h light:12 h dark). At the end of the dark period stock cultures were diluted to a cell density of $1 \cdot 10^6$ cells/ml. Light intensity was 370 $\mu E\ m^{-2}\ s^{-1}$ photosynthetically active radiation. Algae were harvested at $1900 \times g$ and washed twice with 50 mmol/l MES, 0.1 mmol/l $CaCl_2$ buffer at pH 6.5. For the NMR measurements the final pellet was resuspended at a cell density of 10% total intracellular volume in cold buffer and stored at 0–4 °C until used (15–30 min). Cell number and mean cell volume were determined using a Coulter Counter Channelyzer. Chlorophyll content was determined after extraction with 80% acetone (Porra et al. 1989).

To measure the oxygen consumption during a typical NMR experiment the cell suspension was divided into two aliquots. One part was used for the NMR measurement (see below). The parallel suspension was maintained under the same gas supply and temperature conditions (20 °C) and the O_2 consumption rate was determined with a Clark-type O_2-electrode which was fitted into a 20 mm o.d. NMR tube. Iodoacetate (Sigma) was used as an irreversible inhibitor of GAPDH (Harris and Waters 1976) to obtain information about the contribution from the GAPDH/PKG reaction to the measured P_i-ATP exchange. Iodoacetate inhibited cells were supplied with 20 mmol l^{-1} acetate as an external carbon source 30 min before the addition of 1 mmol l^{-1} iodoacetate. In this case the NMR acquisition was initiated after a 90 min waiting time to ensure sufficient time for inhibitor uptake and enzyme inhibition. In regular intervals of 2 hours, 200 μmoles acetate were injected into the 10 ml cell suspension to compensate for the cellular acetate uptake. The O_2 consumption rates of acetate respiring cells both with and without 1 mmol l^{-1} iodoacetate showed no significant difference.

NMR *measurements*

^{31}P in vivo NMR spectra were recorded on a Bruker AM 360 WB spectrometer operating at 145.78 MHz with a flip angle of 90° and a repetition time of 4 s, unlocked and without proton decoupling. The hardware and soft-

ware of the decoupler channel were used to perform the selective saturation by irradiation of a second frequency (CW-signal). Amplification to 13 mW with a Bruker BSV 3 BX equipped with a selective 145.78 MHz power amplifier was used for complete saturation of either the γ-ATP or the P_i signal. Direct off-resonance saturation of those signals, to which the saturated spins are transferred by chemical exchange, can occur. The degree of direct saturation depends on the power of the CW-signal and the separation of the observed resonance from the saturated signal. The effect of direct saturation was corrected by comparison with control spectra, M_{con}. The control irradiation was applied at an equidistant frequency on the opposite side of the observed resonance. In this way it will experience the same direct saturation as in the former spectra with saturated γ-ATP or P_i signal.

During the interpulse delay the cell suspension was bubbled with 100% O_2 for 1.2 s. Accumulation of spectra took about 8 h. During this period the algae showed nearly constant signal intensities. To compensate for even slight metabolic changes during the measurements, spectra of the transient saturation transfer experiments were recorded in the block-averaging mode with 200 transients per block until a sufficient signal-to-noise ratio (S/N ratio) was obtained (Sianoudis et al. 1985). Each acquisition cycle included a spectrum without CW-signal for the determination of the metabolite concentrations.

Prior to Fourier transformation of the free induction decay an exponential multiplication with 30 Hz was applied. All chemical shifts are referenced to 85% H_3PO_4. The signals were fully relaxed and signal intensities were determined with the deconvolution routine of the WIN-NMR software package (Bruker-Franzen, Bremen, Germany). Metabolite concentrations were determined from 5 independent measurements by comparing the signal intensities with the intensity of a calibrated external standard of methylene-diphosphonic acid (MDP). Signal intensities of spectra with saturation were normalized to the corresponding spectra with control irradiation.

Data analysis

In the two-site-exchange network

$$P_i \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ATP \qquad (1)$$

the phosphate group is transferred between P_i and γ-ATP, with k_1 and k_{-1} being the pseudo-first order rate constants. The modified Bloch equations describe the time dependence of the z-magnetizations

$$\frac{dM_{\gamma\text{-ATP}}}{dt} = \frac{M_{0\gamma\text{-ATP}} - M_{\gamma\text{-ATP}}}{T_{1(\gamma\text{-ATP})}} + k_1 M_{P_i} - k_{-1} M_{\gamma\text{-ATP}} \qquad (2a)$$

$$\frac{dM_{P_i}}{dt} = \frac{M_{0P_i} - M_{P_i}}{T_{1(P_i)}} - k_1 M_{P_i} + k_{-1} M_{\gamma\text{-ATP}} \qquad (2b)$$

with M_{0x} denominating the equilibrium magnetization of metabolite x, M_x the instantaneous magnetization and $T_{1(x)}$ the longitudinal relaxation time of this metabolite. Owing to the saturation of a signal e.g. γ-ATP the magnetization $M_{\gamma\text{-ATP}}$ vanishes. Introducing the constant $1/\tau_{P_i} = (1/T_{1(P_i)}) + k_1$, which describes the apparent longitudinal relaxation time in the presence of chemical exchange (Mann 1977), (2b) has the solution

$$\frac{M_{P_i}(t)}{M_{0P_i}} = 1 - k_1\tau_{P_i}(1\text{-}\exp(-t/\tau_{P_i})). \qquad (3)$$

For long saturation times the instantaneous magnetization reaches as new steady state

$$\frac{M_{P_i}(t \to \infty)}{M_{0P_i}} = 1 - k_1\tau_{P_i} \qquad (4)$$

The parameters k_1 and τ_{P_i} can be determined from one set of magnetization transfer data when a transient saturation transfer experiment is performed (Nunnally and Hollis 1979, Degani et al. 1985, Spencer et al. 1988). The values for τ_{P_i} and k_1 were obtained from the non-linear two-parameter-fit to Eq. (3) with the Marquardt algorithm (P.FIT 5.1, Biosoft, Milltown, NJ, USA) using the experimental data measured for a sequence of saturation times. Standard errors were calculated from the diagonal elements of the covariance matrix which was developed during the process of curve fitting. The P_i concentration c_{P_i} and the rate constant k_1 were used to determine the unidirectional flux towards ATP synthesis which is given by

$$F_{P_i \to \gamma\text{-ATP}} = k_1 c_{P_i} \qquad (5)$$

The analysis of the reverse reaction is straightforward.

Alternatively, τ_{P_i} can be measured by an inversion-recovery experiment with selective saturation of the γ-ATP peak (Mann 1977). The independent determination of the steady state value $M_{P_i}(t \to \infty)$ in a steady state saturation transfer experiment enables one to calculate the exchange rate k_1. However, this method is more time consuming and lacks the advantage of obtaining the parameters from one data set. This is important with respect to the time required for signal accumulation in algal suspensions (~ 1000 transients/spectrum) in comparison with yeast (Alger et al. 1982) or perfused organs (Kingsley-Hickman et al. 1987) with only 100 transients/spectrum. Magnetization transfer in living organisms has been reviewed comprehensively by Alger and Shulman (1984) and Brindle (1988 a).

Results

Saturation of the γ-ATP signal in respiring cells of *Chlamydomonas reinhardtii* in the dark resulted in a decline of the P_i intensity (Fig. 1 A, B). The difference between the control and saturated spectrum (Fig. 1 C) indicated the intensity change of P_i^{int}. Intensity changes can also be detected for β-ATP, α-ATP and the phosphodiester (PDE) signals. The decrease of the β-ATP signal resulted from the simultaneous saturation of the β-phosphate group of ADP which has nearly the same resonance frequency as γ-ATP. When ADP is phosphorylated, the saturated β-phosphate of ADP is converted to β-ATP.

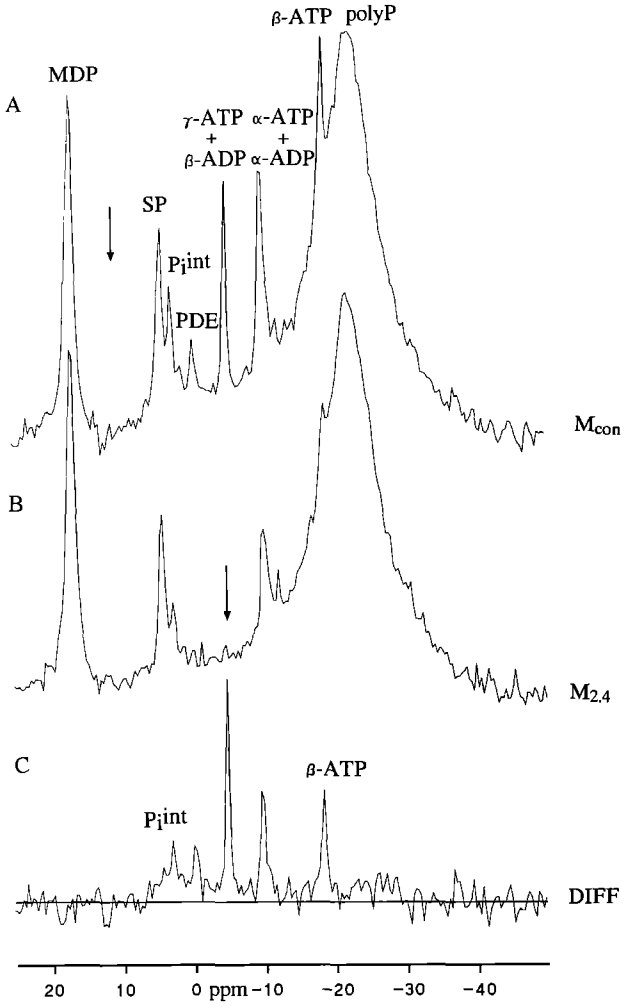

Fig. 1. ^{31}P in vivo NMR spectra of *Chlamydomonas reinhardtii* under aerobic conditions in the dark with control irradiation **A** and with saturation of the γ-ATP signal for 2.4 s ($M_{2.4}$) **B** as indicated by the arrows. Control irradiation occurs equidistantly to the P_i signal on the downfield side. The difference spectrum **C** shows intensity changes of the P_i^{int}, PDE, α-ATP and β-ATP signals. The baseline is indicated in panel **C**. MDP: methylene-diphosphonic acid; SP: sugar phosphates; P_i^{int}: intracellular orthophosphate; α, β, γ-ATP: α, β, γ phosphate groups of ATP; α, β-ADP: α, β phosphate groups of ADP; polyP: polyphosphate

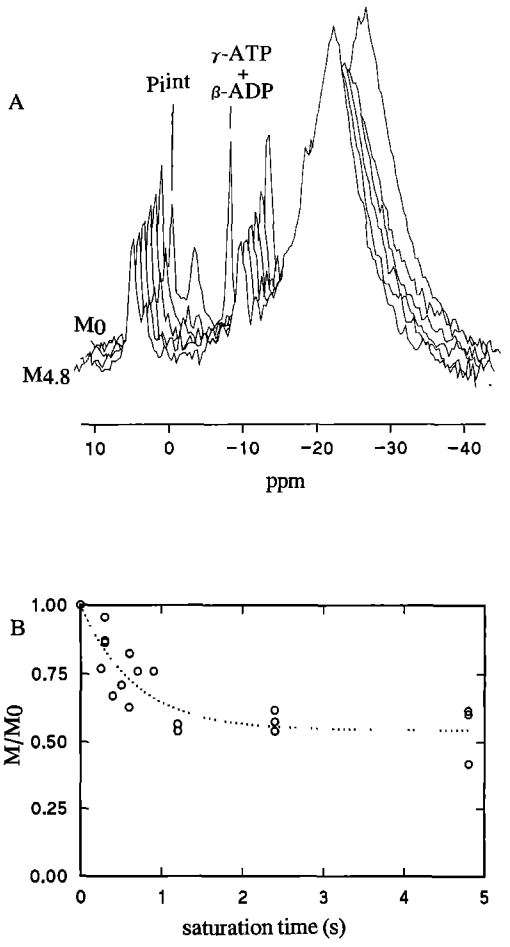

Fig. 2. Series of ^{31}P in vivo NMR spectra **A** of *Chlamydomonas reinhardtii* (*dark, aerobic*) showing the decline of the P_i^{int} signal due to saturation transfer from γ-ATP with saturation times varied between 0.0 s (M_0), 0.3 s, 0.6 s, 1.2 s, 2.4 s and 4.8 s. The instantaneous magnetization of P_i^{int} relative to M_0 is shown as a function of the saturation time in **B**. The *dotted line* represents the two-parameter-fit to (3). Abbreviations as in Fig. 1

Therefore, no magnetization is delivered to this pool. The decline of the intensity of the β-ATP signal is caused by the sum of all ATP consuming reactions and indicates the total flux out of the ATP pool.

The spectral proximity of the α-ATP to the saturated γ-ATP signal is the reason for its appearance in the difference spectrum (Fig. 1 C), owing to off-resonance saturation. This was proved by a control irradiation placed equidistantly at the highfield side of the α-ATP signal. Also the PDE signal which has nearly the same spectral separation from γ-ATP was affected by off-resonance saturation. The saturation of the broad hump underlying the PDE peak occurred nearly independent of the frequency of the CW-signal. This suggests that phospholipids, which experience motionally not averaged chemical shift anisotropy (Murphy et al. 1989), contribute to this signal. Indeed, off-resonance irradiation has been used to eliminate the broad component of the PDE signal (Gonzalez-Mendez et al. 1984). Consequently, it was not necessary to extend the reaction network represented in (1) and we could evaluate the saturation transfer between P_i and γ-ATP directly.

Figure 2 A shows ^{31}P in vivo NMR spectra of *Chlamydomonas reinhardtii* under aerobic conditions in the dark with saturation of the γ-ATP signal in a transient saturation transfer experiment. The data of four independent experiments are compiled in Fig. 2 B. The values for τ_{P_i} and k_1 derived from the two-parameter-fit were used to determine a unidirectional flux towards ATP synthesis of 1.6 ± 0.5 μmol (ml cell volume)$^{-1}$ s^{-1} (Table 1).

Under aerobic conditions in the dark the main contribution to the net ATP synthesis arises from oxidative phosphorylation by the mitochondrial F_0F_1-ATPase. The P/O ratio, defined as the amount of phosphate trans-

reaction	rate constant k s^{-1}	τ_1 s	concentration µmol ml^{-1}	flux µmol $ml^{-1}s^{-1}$	P/O-ratio
$P_i \rightarrow \gamma$-ATP [1]	0.68 ± 0.10	0.67 ± 0.13	2.3 ± 0.7	1.6 ± 0.5	22
$P_i \rightarrow \gamma$-ATP [2] chloroplast	0.68 ± 0.10	0.67 ± 0.13	4.9 ± 1.2	3.3 ± 1.2	n.d.
$P_i \rightarrow \gamma$-ATP [3] iodoacetate	$\leq 0.04 \pm 0.01$	0.79 ± 0.09	9.7	≤ 0.37	~ 2
γ-ATP $\rightarrow P_i$ [1] reverse reaction	1.2 ± 0.4	0.23 ± 0.09	2.8 ± 0.8	3.3 ± 1.5	n.d.
β-ATP $\rightarrow \beta$-ADP [3] total ATP-hydrolysis	1.6 ± 0.5	0.25 ± 0.04	2.8 ± 0.8	4.5 ± 1.8	n.d.

[1] data derived from transient saturation transfer experiments
[2] calculated assuming that P_i is distributed between chloroplast and remaining cell interior in the same ratio as determined by Klöck and Kreuzberg (1991)
[3] data derived from steady state saturation transfer experiments in combination with inversion-recovery experiments for τ_1 determination
n.d.: not defined

Table 1. Undirectional fluxes through ATP synthesizing and consuming reactions. Fluxes in respiring *Chlamydomonas reinhardtii* in the dark were calculated from rate constants k determined by different saturation transfer experiments and the corresponding substrate concentration. Longitudinal relaxation times τ_1 in the presence of chemical exchange and apparent P/O ratios are indicated

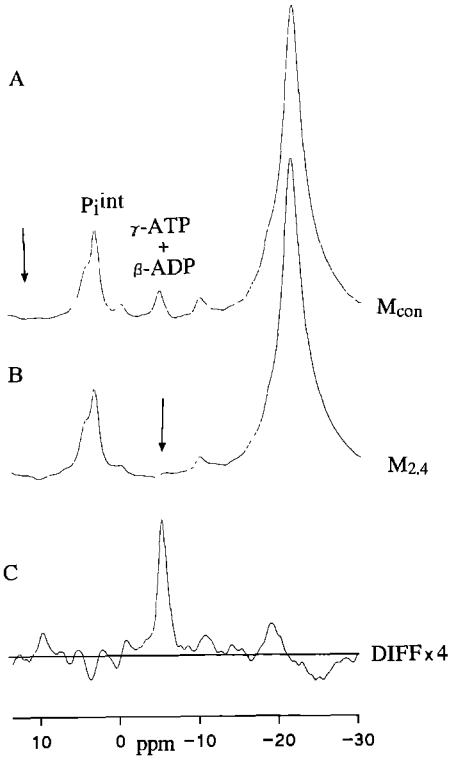

Fig. 3A–C. ^{31}P in vivo NMR spectra of *Chlamydomonas reinhardtii* (*dark, aerobic*) in the presence of iodoacetate. Saturation transfer to P_i^{int} was below the level of detection as can be seen by comparing the spectra **A** with control irradiation M_{con} and **B** with saturation of γ-ATP in the difference spectrum **C**

ferred to ATP divided by the amount of atomic oxygen consumed, is known to be 3 for the mitochondrial F_0F_1-ATPase (Beevers 1960). Saturation transfer experiments, however, determine unidirectional fluxes. These fluxes can also arise from enzymes working near equilibrium, thereby catalyzing high unidirectional fluxes in both directions with only small net fluxes. The cell suspensions which were used for the saturation transfer experiments of Figs. 1 and 2 showed an oxygen consumption rate of 0.036 µmol O_2 (ml cell volume)$^{-1}$ s^{-1}, resulting in an apparent P/O ratio of 22. To identify the enzymes which catalyze the measured flux in *Chlamydomonas* we inhibited GAPDH activity by iodoacetate and evaluated again the P/O ratio.

A steady state saturation transfer experiment under aerobic conditions in the dark performed on iodoacetate treated *Chlamydomonas reinhardtii* cells respiring on external acetate is shown in Fig. 3 in comparison with the control spectrum M_{con}. Despite the clearly elevated P_i^{int} signal no saturation transfer to this signal could be detected in the difference spectrum (Fig. 3C). Taking the noise intensity of the difference spectrum as an upper limit for the saturation transfer, the decline of the magnetization has to be smaller than 0.03. In this special case the difference between the intrinsic longitudinal relaxation time $T_{1(P_i)}$ and the apparent relaxation time τ_{P_i} could be neglected as no chemical exchange was observed in the spectra of Fig. 3. Therefore the $T_{1(P_i)}$ derived from an inversion recovery experiment without additional irradiation can be used to calculate the rate constant $k_1 \leq 0.04\ s^{-1}$ (Table 1). After iodoacetate treatment, 0.115 µmol O_2 (ml cell volume)$^{-1}$ s^{-1} were consumed by acetate respiring cells, resulting now in an apparent P/O ratio of about 2.

A complete description of the exchange network also requires the determination of the flux from ATP towards P_i. This flux must equal the flux towards ATP synthesis, because metabolite concentrations are in a steady state under constant physiological condition. This experiment also tests the assumption of a two-site exchanging network and examines whether it is correct to use the mean intracellular metabolite concentration for flux calculation, ignoring subcellular compartmentation.

The direct measurement of the flux from ATP to P_i was achieved by saturating the P_i signal and following the decline of the γ-ATP intensity in a transient saturation transfer experiment (Fig. 4A). The two-parameter fit (Fig. 4B) gave simultaneously $\tau_{\gamma\text{-ATP}}$ and k_{-1}. The flux in the reverse reaction was calculated to $F_{\gamma\text{-ATP} \rightarrow P_i} = 3.3 \pm 1.5$ µmol (ml cell volume)$^{-1}$ s^{-1} (Table 1).

Another estimation of ATP degradation is also possible through the decline of the β-ATP signal, since con-

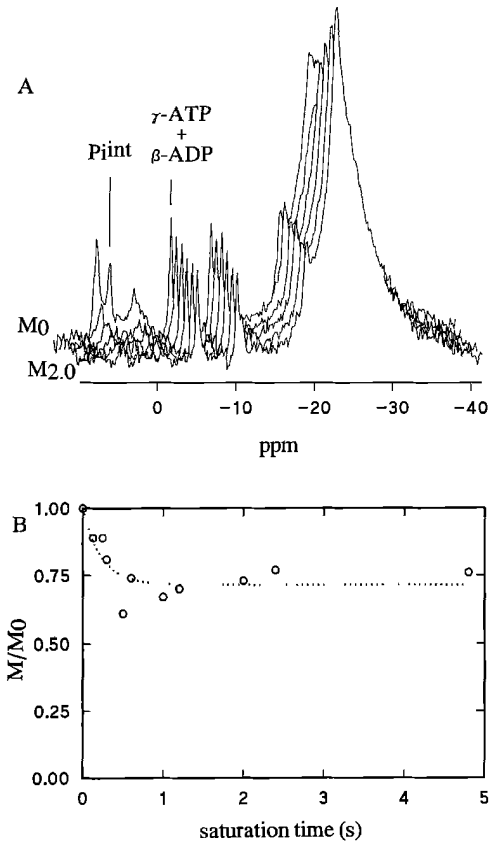

Fig. 4. ^{31}P in vivo NMR spectra of *Chlamydomonas reinhardtii* measured in the dark under aerobic conditions with saturation of the P_i^{int} signal for 0.0 s (M_0), 0.13 s, 0.25 s, 0.5 s, 1.0 s and 2.0 s **A**. The time course of the instantaenous magnetization of γ-ATP relative to M_0 is shown in **B** with the dotted line representing the two-parameter-fit to an equation analogous to (3)

comitantly with the γ-ATP the β-ADP signal is saturated. Such a transient saturation transfer experiment is hampered by the short T_1 of β-ATP and its unfavourable chemical shift close to the broad polyphosphate peak. Therefore, the steady state value of the β-ATP magnetization was determined to $M_{\beta\text{-ATP}}(t \rightarrow \infty)/M_{0\beta\text{-ATP}} = 0.60 \pm 0.12$. The apparent longitudinal relaxation time $\tau_{\beta\text{-ATP}}$ was measured in an inversion recovery experiment with saturation of the γ-ATP peak. From these values the rate constant $k'_{-1} = 1.6 \pm 0.5\ s^{-1}$ and the resulting total ATP hydrolysis $F_{\beta\text{-ATP} \rightarrow \beta\text{-ADP}} = 4.5 \pm 1.8$ μmol (ml cell volume)$^{-1}$ s^{-1} could be calculated (Table 1). This flux, however, also includes all reactions which transfer the γ-phosphate group of ATP to other pools than P_i e.g. the reaction catalyzed by the adenylate kinase, which is known to operate near equilibrium (Gupta 1979).

Discussion

In isolated plant mitochondria the net flux through the F_0F_1-ATPase towards ATP synthesis yields a P/O ratio of three (Beevers 1960). A similar P/O ratio was also found in studies of ATP synthesis in kidney (Freeman et al. 1983), rat hearts (Matthews et al. 1981) and maize root tips (Roberts et al. 1984) using the unidirectional flux derived from saturation transfer experiments. This coincidence of net and unidirectional fluxes was taken as evidence that the F_0F_1-ATPase operates unidirectionally in vivo and causes the observed magnetization transfer. Because in *Chlamydomonas reinhardtii* the measured apparent P/O ratio of 22 was much larger than the canonical value of 3, the F_0F_1-ATPase can not be alone responsible for the observed flux towards ATP. Similar high P/O ratios have been observed by saturation transfer experiments performed on *Saccharomyces cerevisiae* (Alger et al. 1982), *E. coli* (Mitsumori et al. 1988) and rat hearts (Kingsley-Hickman et al. 1986).

Inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with iodoacetate resulted in a distinct decrease of the P/O ratio indicating that GAPDH coupled with PGK caused the observed magnetization transfer in these organisms (Campbell-Burk et al. 1987, Brindle 1988 b, Mitsumori et al. 1988). Also in *Chlamydomonas* iodoacetate strongly decreased the unidirectional flux from P_i to ATP and reduced the P/O ratio to a value of 2.

Photosynthetic eucaryotes usually contain two distinct phosphorylating GAPDH isoenzymes (Mateos and Serrano 1992). The NAD-specific enzyme (EC 1.2.1.12) is located in the cytosol. The other, chloroplastidic GAPDH (EC 1.2.1.13), which is the prevailing enzyme in *Chlamydomonas* (Klein 1986), is active with both NADP and NAD as coenzyme and functions during photosynthetic CO_2 fixation as part of the Calvin cycle (Leegood 1990). Iodoacetate is known to inhibit GAPDH through carboxymethylation of a conserved cysteine residue in the active site (Harris and Waters 1976). Although iodoacetate can interact with a variety of other proteins containing nucleopilic amino acid residues, GAPDH seems to be comparatively sensitive to iodoacetate inactivation. In the green alga *Chlorella pyrenoidosa*, iodoacetate reduced the photosynthetic $^{14}CO_2$ fixation rate and diverted the ^{14}C label from sucrose and starch into 3-PGA and metabolites associated with the TCA cycle (Hiller 1970). This finding suggests that chloroplastidic GAPDH is the principal site of iodoacetate inhibition in vivo, thus blocking the pathway from 3-PGA to hexoses and storage carbohydrates. Furthermore, it was shown in isolated wheat chloroplasts that iodoacetol phosphate, also an inhibitor of GAPDH, did not inactivate the metabolite transfer catalyzed by the phosphate translocator in the chloroplast envelope (Usuda and Edwards 1981).

From this circumstantial evidence and the effect of iodoacetate on *Chlamydomonas* described in this paper we conclude that the magnetization transfer in non-inhibited cells must be attributed to the coupled enzymes GAPDH/PGK. Taking into consideration the distinct compartmentation of GAPDH/PGK in the chloroplast (Klein 1986), most of the observed magnetization transfer has to be attributed to the chloroplast. Necessarily, the involved substrates, including P_i, also have to be located in the chloroplast stroma.

Kingsley-Hickman et al. (1987) emphasized that transport processes across membranes must also be taken into consideration for interpreting magnetization transfer

data in eucaryotic cells. The assignment of the P_i signal to the cytosol may still hold for *Chlamydomonas reinhardtii*, if one assumes rapid exchange of orthophosphate between the chloroplastidic and cytosolic compartment with respect to its spin-lattice relaxation time. Saturation-labelling of P_i may occur in the chloroplast stroma followed by an immediate transport of P_i to the cytosol where the decline of the magnetization is detected. P_i exchange between chloroplast and cytosol is mediated by the phosphate translocator, whose transport kinetics have been well characterized in higher plant chloroplasts (Fliege et al. 1978) and which was also identified in *Chlamydomonas reinhardtii* (Klein et al. 1983). The translocator acts as an antiport, transferring phosphate to the chloroplast in exchange for phosphorylated sugars, mainly triose-phosphates and 3-PGA, and vice versa.

The maximum transport rate of the phosphate translocator in intact spinach chloroplasts at 20°C was about 250 μmol (mg Chl)$^{-1}$ h^{-1} (Fliege et al. 1978). Assuming similar transport characteristics in *Chlamydomonas* chloroplasts (Klein et al. 1983), a hypothetical rate for P_i export from the chloroplast can be calculated (Lilley et al. 1977). Taking the chlorophyll content of 16.2 ± 2.5 mg (ml cell volume)$^{-1}$ ($n = 5$) and using the chloroplastidic metabolite levels for the competing substrates P_i, triose phosphate and 3-PGA determined by Klöck and Kreuzberg (1991), the P_i export from the chloroplast would be 0.6 μmol (ml cell volume)$^{-1}$ s^{-1}. Even assuming (i) a complete saturation of the chloroplastidic P_i pool by saturation transfer from γ-ATP ($k_1 \gg \tau_{P_i}^{-1}$) and (ii) an exclusion of any reverse flow of saturated cytosolic P_i to the chloroplast, the decline of the P_i magnetization would be limited to about 17%. This is in contradiction to the requirement of rapid transport stated above, because it would take at least 5.8 times the spin-lattice relaxation time for complete exchange of P_i. Consequently, the observed magnetization transfer cannot be explained by assuming an exclusive localization of the NMR visible P_i in the cytosol. Rather most of the P_i signal intensity has to be assigned to the chloroplast. This conclusion is also supported by the recent NMR detection of P_i in isolated spinach chloroplasts (Bligny et al. 1990) as well as by the high amount of chloroplastidic P_i (85% of total cellular orthophospate) in *Chlamydomonas* (Klöck and Kreuzberg 1991).

Until recently it was suggested that the mitochondrial phosphate signal is NMR invisible (Bailey et al. 1981). However, in a study on the NMR visibility of inorganic phosphate in isolated rat liver mitochondria it was shown that matrix P_i is mostly observable by NMR under physiological conditions (Hutson et al. 1992). Indeed, two P_i signals have been observed with ^{31}P-NMR spectroscopy in isolated, perfused rat hearts (Garlick et al. 1983). Further determination of the myocardial mitochondrial P_i content using density gradient centrifugation in non-aqueous solvents and quantitative electron microscopy proved, that the more alkaline P_i signal originates from P_i within the mitochondria (Garlick et al. 1992).

The concentration of a substrate available to an enzyme may be different from the mean intracellular concentration, if there is compartmentation. In this case it is important to assess the intracellular distribution of the substrate (Brindle 1988 a, Loughman et al. 1989). Because the cytosol accounts for only 40% of the total cell volume in *Chlamydomonas* (Schötz et al. 1972), the assignment of the complete P_i signal exclusively to the cytosol would result in a cytosolic concentrations of 5.8 μmol (ml cytosol volume)$^{-1}$. However, extraplastidic P_i concentrations above 1 μmol ml^{-1} have been shown to inhibit photosynthetic CO_2 fixation in isolated chloroplasts by the depletion of stromal triose phosphate in exchange for P_i (Flügge et al. 1980, Klein et al. 1983).

However, if we assume equal NMR visibility of P_i both in the chloroplast and in the cytosol and if we use the data of Klöck and Kreuzberg (1991) for the intracellular distribution of P_i, we obtain a P_i concentration in the chloroplast of 4.9 ± 1.2 μmol (ml chloroplast volume)$^{-1}$ and a flux through the GAPDH/PGK reaction towards ATP synthesis of 3.3 ± 1.2 μmol (ml chloroplast volume)$^{-1}$ s^{-1} (Table 1). According to Klöck and Kreuzberg (1991) there is no difference in the concentration of ATP in the chloroplast and in the cytosol. The ATP concentration determined by ^{31}P in vivo NMR can therefore be used to calculate the flux in the reverse reaction from γ-ATP to P_i. This flux was nearly identical with the unidirectional ATP synthesis in the chloroplast provided the compartmentation of P_i is taken into consideration (Table 1). This indicates again that the restriction to a two-site-exchange network is sufficient and that the GAPDH/PKG reaction operates near equilibrium. Similar results were observed in *E. coli* (Mitsumori et al. 1988) and *S. cerevisiae* (Brindle and Krikler 1985) where it was shown also that the flux control coefficient of these enzymes for the glycolytic flux was very low (Brindle 1988 b).

The fact that chloroplastidic P_i can be detected by ^{31}P in vivo NMR and accounts for most of the P_i signal in *Chlamydomonas* is of special importance for the use of the chemical shift of the P_i signal as a pH-marker and for the interpretation of intracellular pH as a regulator in plant cell metabolism.

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