^{31}P NMR STUDIES OF PHOTOPHOSPHORYLATION IN INTACT CELLS OF *CHROMATIUM VINOSUM*

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1. Introduction

^{31}P NMR contributed significantly to our understanding of the bioenergetics of intact cells and organs (review [1,2]). Being a non-invasive technique ^{31}P NMR allows direct determinations of the concentrations of phosphorylated metabolites within intact cells and tissues [3–5] and gives information about their cellular environment [6]. In addition it is extremely powerful in observing possible intracellular compartmentation [7–9] and in establishing heterogeneity of the cellular system, especially with respect to internal pH [10–12].

In [13,14], we reported the application of ^{31}P NMR to study energy transduction in *Rhodopseudomonas sphaeroides*. In this phototrophic purple non-sulfur bacterium an homogeneous intracellular compartment was revealed [13] and the magnitude of the light-induced transmembranal pH gradient could be quantitatively established [14]. No evidence for a distinct intrachromatophore compartment was obtained. In spite of the fact that adenine nucleotides could be demonstrated chemically [13], no ADP and ATP resonances could be observed in ^{31}P NMR spectra of *Rps. sphaeroides* cells. Here we extend our studies to the phototrophic purple sulfur bacterium *Chromatium vinosum*. By extraction methods it has been shown that *Chr. vinosum* contains high levels of adenine nucleotides [15]. This bacterium is assumed to have a distinct intrachromatophore space, which is membrane-separated from the intracytoplasmic aqueous phase [16]. However, from our ^{31}P NMR studies no evidence for intracellular compartmentation in *Chr. vinosum* has been obtained.

^{31}P NMR spectra of suspensions of this organism reveal high levels of nucleotides. The concentrations of nucleoside di- and triphosphates have been measured under dark and photophosphorylating conditions. On the basis of these measurements estimations of the internal phosphorylation potential and energy charge are made. Under our experimental conditions the maximal phosphorylation potential in *Chr. vinosum* under light-energized conditions is 10.3 kcal/mol.

Abbreviations: ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential gradient; DCCD, *N,N'*-dicyclohexylcarbodiimide; NMP, NDP, NTP, nucleoside mono-, di- and triphosphates, respectively

2. Experimental

2.1. *Growth and harvesting of cells*

Chromatium vinosum, strain DSM 185, was grown anaerobically at saturating intensities of incandescent light, in the medium of [17] except that it was supplemented with 10 mM acetate. Doubling the carbonate buffer content effectively restricted pH changes during growth to ⩽0.4 pH unit.

Cells were harvested after 4 days and washed 5–10 times at room temperature with a medium containing 50 mM KCl, 5 mM $MgSO_4$, 2 mM K-EDTA, 5 mM KH_2PO_4, 30 mM 2-[*N*-morpholino] ethanesulphonic acid, 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid, supplemented with 10 mg chloramphenicol/l. Finally, the cells were washed and resuspended with the same medium except that 10% ^2H_2O was added and potassium phosphate was omitted. The cell density was typically 70–100 mg protein/ml.

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2.2. *^{31}P NMR measurements*

^{31}P NMR measurements (145.78 MHz) were performed essentially as in [13] except that spectra were accumulated with a recycle time of 0.34 s and a spectral width of 6000 Hz. 45° radio frequency pulses were employed.

Glycerophosphorylcholine (2 mM) was usually added to the bacterial suspension. It is known to resonate at −0.49 ppm from 85% orthophosphoric acid and was used for the calibration of chemical shifts of orthophosphates to determine the intra- and extracellular pH [12–14] and for the calibration of internal metabolite concentrations. The partial saturation of resonances originating from the short recycle time was corrected by comparison with fully relaxed spectra.

Illumination (350 mW) in the NMR probe was provided by an argon ion laser as in [13].

2.3. *Protein determination*

Protein was determined according to [18], after boiling of the cells for 2 min in 2 M NaOH. At the time of harvesting the cells did not contain elemental sulfur. The latter component is known to interfere with the Lowry estimation of protein.

2.4. *Internal volume of the cells*

The internal volume of the cells was determined to be 2 μl/mg protein with the use of $^{3}H_2O$ and [*carboxy*-^{14}C]dextran as in [14].

2.5. *Materials*

All materials were reagent grade and obtained from commercial sources.

3. Results

Fig.1 shows representative ^{31}P NMR spectra of a dense suspension of *Chromatium vinosum* cells incubated in the dark. An interesting feature displayed in these spectra is the presence of two strong resonances (P_i and P_x) in the orthophosphate chemical shift region. Although of variable intensity, the peak labeled P_x is present among different cell preparations; its area shows only minor changes under different metabolic conditions. By ^{31}P NMR experiments on a perchloric acid extract of *Chr. vinosum* cells it was established that peak P_i represents intracellular inorganic phosphate. In the perchloric acid extract peak P_x shows a constant shift of −1.35 ppm in the physiological pH range. From the chemical shift of the resonance labeled P_i we estimate that the intracellular pH after 2 h incubation in the dark is 7.24 (fig.1A). Fig.1A shows a high 'dark' NTP/NDP ratio of 3.7. Upon prolonged incubation in the dark (fig.1B) the cells have a slightly higher P_i level paralleled by a decrease in the NTP/NDP ratio to 2.0. Other metabolite levels remain constant. Internal pH decreases slightly to pH 7.18 as judged from the P_i chemical shift position in fig.1B. The chemical shifts of the internal NDP and NTP peaks indicate that these nucleotides are mostly in

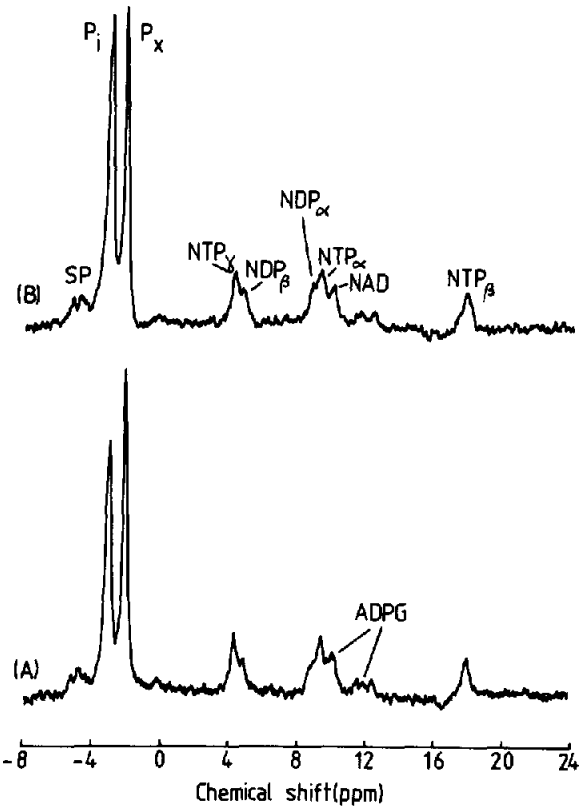

Fig.1. ^{31}P NMR (145.78 MHz) spectra of a cell suspension of *Chromatium vinosum*. Cells were grown for 4 days and harvested as in section 2. The pH of the suspension medium was 7.2. Each spectrum represents the time average of 5000 scans. Spectra: (A) from a suspension incubated for 2 h in the dark after the final resuspension; (B) from the same preparation as A after 4 h dark incubation. Peaks were assigned on the basis of chemical shifts. SP, sugar phosphates; P_i, intracellular orthophosphate; P_x, not assigned; NTP_γ, NTP_β, NTP_α, terminal, middle and primary phosphates of nucleoside triphosphates, respectively; NDP_β, NDP_α, terminal and primary phosphates of nucleoside diphosphates, respectively; NAD, nicotinamide dinucleotide; ADPG, adenine diphosphoglucose.

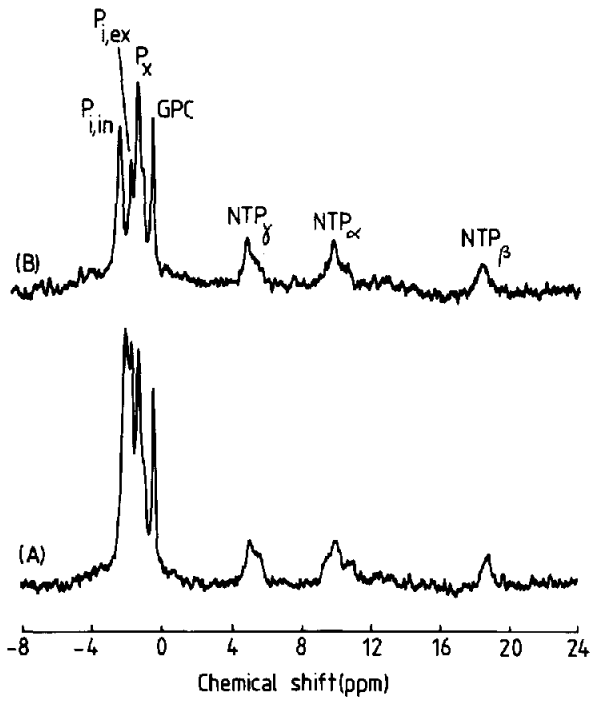

Fig.2. ^{31}P NMR spectra of a suspension of *Chromatium vinosum* cells. For each spectrum 2000 scans were averaged. After the final resuspension 2 mM glycerophosphorylcholine (GPC) and 0.5 mM potassium phosphate ($P_{i,ex}$) were added. Spectra: (A) from a suspension stored in the dark for 8 h; (B) taken after 35 min illumination in the NMR probe. Other conditions and symbols are as in fig.1 except that $P_{i,in}$ represents intracellular orthophosphate.

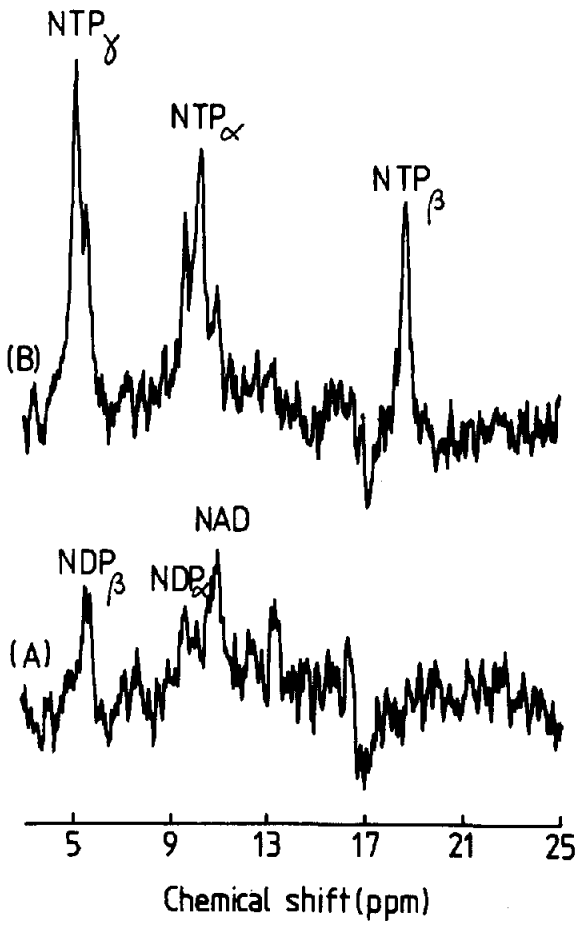

Fig.3. Di- and trinucleotide region of ^{31}P NMR spectra of *Chromatium vinosum* cells. Cells were harvested and washed as in section 2. For both spectra 2000 scans were accumulated. Spectra: (A) from a suspension stored for 4 days at 4°C in the dark; (B) from the same preparation taken after 47 min steady-state illumination (350 mW). Other conditions and symbols as fig.1.

the Mg^{2+}-bound form [19]. Fig.2 demonstrates the effect of illumination on *Chr. vinosum* cells after 8 h dark storage. Fig.2A represents the dark control. After ~25 min illumination the internal pH has risen from pH 7.01 to the steady-state value of 7.29; the ΔpH increases from 0.38–0.70. The addition of 20 μM valinomycin leads to an increase of the light-induced ΔpH to 0.88 (not shown). The NTP/NDP ratio increases from 1.6–2.5 upon illumination. The $P_{i,in}$ concentration declines correspondingly in the light (fig.2B).

Storage of the cells for 4 days in the dark at 4°C completely depleted the cells of NTP leaving only a low level of NDP (fig.3A). Illumination initiates photophosphorylation leading to almost complete restoration of the NTP/NDP ratio (fig.3B) as observed in the original suspension (fig.1A). The free NDP concentration remains essentially unchanged upon light energization. Hence, the NTP signal produced in the light is much higher than the NDP signal originally present in the dark. The amount of NMP in the dark is quite similar to the level present under energized conditions (not shown). Therefore, the increased level of internal NTP plus NDP during illumination cannot be explained by phosphorylation of internal NMP. Fig.3B elegantly demonstrates the remarkable shift resolution for the terminal and primary phosphate resonances of NDP and NTP. Such a result could only be obtained after repeated washings of the cells with medium containing EDTA to remove paramagnetic ions from the cells (see also [13]).

In *Chr. vinosum* phosphorylation in the light can

be inhibited by DCCD (fig.4). The decrease in the NTP/NDP ratio during starvation (see fig.4A) can be reversed by illumination of the suspension (see fig.4B). The presence of 0.5 mM DCCD, however, inhibits this for 90% (fig.4C). Clearly, higher DCCD concentrations are needed to block the ATP-synthetase reaction completely. The NTP and NDP levels in the dark in the DCCD-treated and untreated suspension are the same (not shown) indicating that ATP hydrolysis via the membrane-bound ATPase complex is unimportant under these conditions. In the light polyphosphate is also synthesized (fig.4C) as judged from the appearance of a ^{31}P NMR resonance at 23.1 ppm. Obviously, this process is also influenced by DCCD since this peak does not appear in the inhibitor-treated suspension. It is of interest that DCCD does not influence the generation of a light-induced ΔpH across the cytoplasmic membrane: ΔpH at steady-state in the light is 0.77 and 0.73 for the DCCD-treated and control suspension, respectively (at an external pH 6.0) [20].

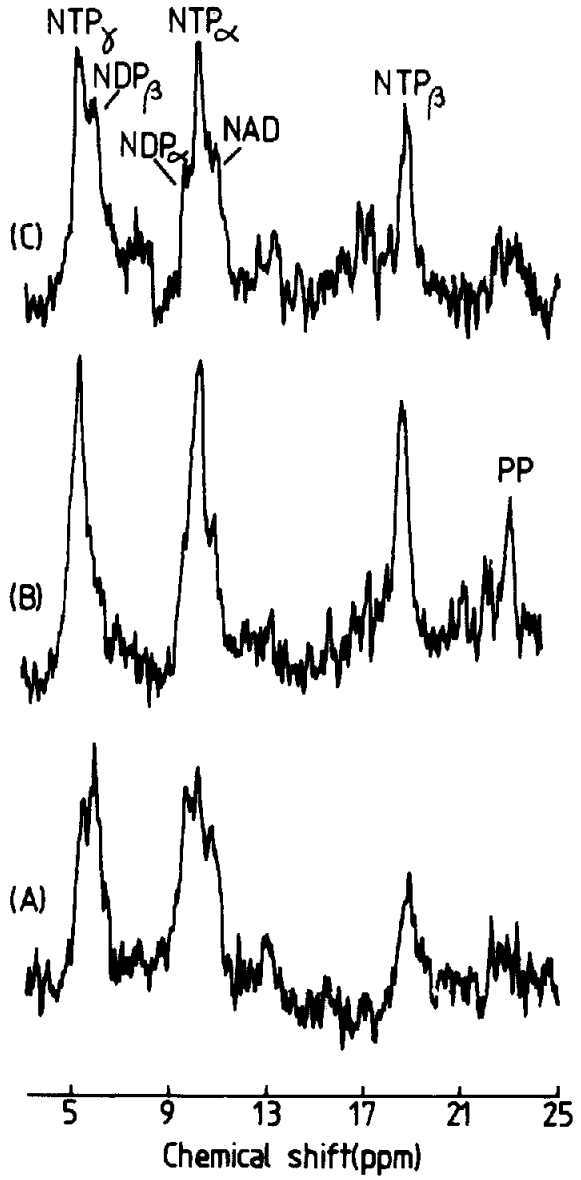

Fig.4. Di- and trinucleotide region of ^{31}P NMR spectra of *Chromatium vinosum* cells. After harvesting and washing the cells were stored at 4°C in the dark for 2 days. Each spectrum represents the time average of 2000 scans. Spectra: (A) taken in the dark; (B) spectrum of cells illuminated for 33 min; (C) spectrum of cells taken after 45 min steady-state illumination in the presence of 0.5 mM DCCD; PP, middle peaks of polyphosphate chains. Other symbols and conditions as in fig.1.

4. Discussion

Many of the important questions concerning the bioenergetics of whole cells and intact organs will only be resolved when we are able to determine the parameters involved under physiological conditions. This report clearly demonstrates that ^{31}P NMR is an attractive method for an in vivo approach in the study of the energy balance in phototrophic procaryotes.

These data show that *Chromatium vinosum* is capable of maintaining a highly energized state for long dark periods (fig.1,2). Previous measurements with intact cells revealed a 90% viability after 2 days dark incubation while after 4 days as much as 70% of the cells are able to multiply [15]. Moreover, the organism's viability is explained by a fast recovery of high NTP levels through photophosphorylation (fig.2–4). Light-induced proton translocation across the cytoplasmic membrane creates a pH gradient (internal basic) in *Chr. vinosum* ranging from 0.7–0.8 at an external pH 6.0–6.4. Since no attempts have been made to measure the maximal ΔpH at light saturation these values are certainly suboptimal [12,14].

The internal P_i concentration, estimated from the ^{31}P NMR spectra ranges from 20–70 mM among different cell preparations. Under light-energized conditions the internal NTP concentration can amount up to 15 mM corresponding to ~30 nmol NTP/mg protein. Previous determinations of the ATP content of *Chr. vinosum* by extraction methods [15] led to maximal levels of 10 nmol ATP/mg protein. This means that

either ATP contributes only for 30% to the total trinucleotide content of *Chr. vinosum* or the procedures commonly employed lead to incomplete extraction, thereby resulting in a severe underestimation of internal ATP concentrations.

From the observed concentrations of internal P_i, NDP and NTP an estimation for the intracellular phosphorylation potential can be made if one assumes that the ATP/ADP ratio is proportional to the observed NTP/NDP ratio. The latter assumption seems justified as it has been reported that the levels of uracil, cytosine and guanine nucleotides in *Chr. vinosum* respond to changes in metabolic conditions just as do the adenine nucleotides [21]. Besides the levels of P_i and the NTP/NDP ratio also the cellular free Mg^{2+} concentration and internal pH have to be known to calculate the internal ATPase free energy. The lower limit to the free Mg^{2+} concentration can be set to ~3 mM on the basis of the observation that all detectable NTP is bound to Mg^{2+} while an upper limit is given by the total intracellular concentration which was found to be 20 mM [22]. At an internal pH 6.7 the standard free energy of ATP hydrolysis is 6.67 and 6.66 kcal/mol for a free Mg^{2+} concentration of 3 and 20 mM, respectively [23].

At an ATP/ADP ratio of 10.9 the free energy of ATP hydrolysis would then be 10.3 kcal/mol for both concentrations of free Mg^{2+} mentioned above. This value is observed at 22 mM internal P_i which is calculated assuming that the internal volume of the cells is 2 μl/mg protein. It is, however, not very sensitive to errors in the determination of internal volume since at an internal volume of 4 μl/mg protein the maximal internal ATPase free energy under light-energized conditions would rise to 10.7 kcal/mol. At the highest P_i levels observed (70 mM) the phosphorylation potential determined ranges from 8.8–9.5 kcal/mol under our conditions. The maximal values for the phosphorylation potential in *Chr. vinosum* reported here are similar to ^{31}P NMR results obtained from respiring mitochondria [24,25] and glycolizing, anaerobic *Escherichia coli* [26]. These values, however, are low compared to those published for a suspension of chromatophores. Presumably, in the latter case both the virtual absence of ATP consuming processes and the fact that larger transmembrane gradients can be maintained as has been reported for $\Delta\psi$ in [27] can account for phosphate potentials of up to 15 kcal/mol [28,29] in a suspension containing these subcellular particles.

The energy state of a cellular system is often expressed by the energy charge [30]. From the observed concentrations of NMP, NDP and NTP it is calculated that in *Chr. vinosum* in the light the energy charge has a minimum value of 0.90 while at maximum it becomes as high as 0.97. These values are significantly higher than those in [21,22] who found values of 0.76–0.86. The discrepancy might be explained by an underestimation through the extraction methods employed in the latter studies as acid extraction can give rise to significant hydrolysis of nucleoside triphosphates.

Subcellular compartmentation can be studied by means of ^{31}P NMR. Here no experimental evidence for chromatophore-like intracellular compartments has been found. The internal P_i signal reflects a homogeneous internal pH as has been found for *Rhodopseudomonas sphaeroides* [13]. Similar observations were made with cell suspensions of *Rhodopseudomonas capsulata* and *Rhodospirillum rubrum* (K.N., K.J.H., unpublished). These observations indicate that either the widely held notion that phototrophic bacteria contain intracytoplasmic vesicles [16] is wrong or that these hypothetical compartments do not contain phosphate metabolites in a significant concentration.

The remarkably strong resonance peak P_x probably belongs to a phosphodiester but its origin remains to be elucidated.

The well resolved resonances of nucleotides in ^{31}P NMR spectra of *Chr. vinosum* cells hopefully will allow saturation transfer experiments. By means of this method the in vivo turnover of the terminal phosphate group of adenosine triphosphate can be measured. Such experiments cannot be performed with biochemical methods [31].

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