Chapter 11

Mitochondrial, Acidic, and Cytosolic pHs Determination by ^{31}P NMR Spectroscopy: Design of New Sensitive Targeted pH Probes

Marcel Culcasi, Sophie Thétiot-Laurent, Ariane Atteia, and Sylvia Pietri

Abstract

^{31}P nuclear magnetic resonance (NMR) is a unique technique to monitor noninvasively the energetics of living systems at real time through the detection of a variety of phosphorylated metabolites. Using adequately designed α-aminophosphonates as external probes, we have shown earlier that ^{31}P NMR can also give access simultaneously to the accurate pH of cytosolic and acidic compartments in normal and stressed cultured cells or isolated perfused organs, a feature that was not possible using endogenous inorganic phosphate as the probe. More recently, we obtained a series of derivatives of these new pH probes that incorporate a triphenylphosphonium cation as a specific vector to the mitochondrion. Here, we describe the synthesis, ^{31}P NMR pH titrating properties in buffers, and application in cultures of the green alga *Chlamydomonas reinhardtii* of two of these mitochondria-targeted pH probes in comparison with one nonvectorized, yet still informative α-aminophosphonate.

Key words ^{31}P NMR, pH Probes, Mitochondria-targeted aminophosphonates, Cytosolic pH, Subcellular pH, Mitochondria, Acidic vacuoles, *Chlamydomonas reinhardtii*

1 Introduction

In the three last decades ^{31}P nuclear magnetic resonance (NMR) has become a privileged dynamic strategy to monitor noninvasively the intracellular content of phosphorylated metabolites (i.e., adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine phosphate, sugar phosphates, and inorganic phosphate (P_i)), for example in cells [1, 2] and isolated perfused organs [3]. In this area, most of the studies of the biological events affecting the cytosolic pH have exploited the pH-dependent chemical shift (δ) of the ^{31}P NMR resonance peak of endogenous P_i, the pK_a of which corresponds to the neutral region. However spectroscopic pH probing using P_i is not efficient when addressed to more acidic

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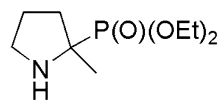
compartments (pH < 6) such as vacuoles in plants and secretory vesicles in mammalian cells or in more alkaline organelles (such as mitochondria or peroxisomes) since P_i is not ^{31}P NMR visible in these compartments due to its very poor subcellular distribution and low spectral sensitivity. Yet using the ^{31}P NMR resonance peak of P_i the mitochondrial pH in the isolated rat liver could be probed, ranging 7.0–7.3, but in these experiments the organs had to be set under hypothermic conditions to increase the P_i content by swelling mitochondria [4].

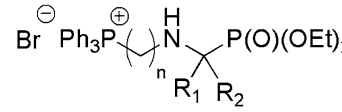
In a more invasive approach to mitochondrial pH determination under normothermic conditions, fluorescent probes were successfully applied in mammalian cells, including human HeLa cells or rat resting cardiomyocytes [5], pancreatic β-cells submitted to glucose stimulation [6], and glutamate-treated rat cortical neurons [7], or in microbes cultures [8] and allowed the determination of cytosolic/mitochondrial pH gradients. In these investigations [5–8] the mitochondrial pH was found in a relatively large range of 6.5–7.9, strongly varying upon cell lines and metabolic or physiopathological conditions.

Because these fluorescent probes have no potential to provide additional information on cell energetics, we have designed a series of nontoxic, linear and cyclic α-aminophosphonates (e.g., DEPMPH (**1**) in the cyclic series; *see* Fig. 1) in which protonation at the amino site leads to a strong structure and pH-dependent variation of the ^{31}P NMR chemical shift at the diethoxyphosphoryl group [9]. Thus, modulating the chemical substituents and the net charge around the N center in these new pH probes allowed to finely tune their subcellular permeation by passive diffusion, the pK_a value (in the range 3–9), and the sensitivity of the ^{31}P NMR detection (as defined by the difference $\Delta\delta_{AB}$ between the ^{31}P chemical shift of the protonated ammonium (δ_A) and non-protonated amine (δ_B) forms), leading to a fourfold increase with respect to P_i (for which $\Delta\delta_{AB} \sim 2.5$ ppm) [10–12].

Despite these α-aminophosphonates successfully probed the pH in cytosol and acidic compartments in constrained cells and isolated perfused organs [10–12], they did not reach the



DEPMPH (1)



Mito-DEP-C8 (**2**): n = 8; $R_1 = R_2$ = Me

Mito-DEP-C4 (**3**): n = 4; R_1 = H, R_2 = iPr

Fig. 1 Structures of ^{31}P NMR pH probes DEPMPH (**1**), mito-DEP-C8 (**2**), and mito-DEP-C4 (**3**)

mitochondria. To overcome this difficulty we have recently developed a series of derivatives bearing a triphenylphosphonium tail, a lipophilic cation that confers selective affinity for the mitochondria [13]. These new mitochondria-targeted α-aminophosphonates were found to keep the attractive ^{31}P NMR sensitivity of their parent molecules (i.e., $\Delta\delta_{AB} \sim 10$ ppm) with an extended pK_a range of 6.0–7.0 [14].

In this paper, we describe a complete methodology for researchers associated with ^{31}P NMR studies on proton and energetic dynamics within the mitochondrion. This includes a full description of the synthesis and analytical features of **1** and two selected mitochondria-targeted compounds, mito-DEP-C8 (**2**) and mito-DEP-C4 (**3**) (Fig. 1); the ^{31}P NMR determination of their spectral characteristics (pK_a, $\Delta\delta_{AB}$) in three media mimicking the cytosolic, acidic vesicles, and mitochondrial environments; and an original study in which the differences between **1** versus (**2**, **3**) towards mitochondrial pH probing were tested in living cultured green alga *Chlamydomonas reinhardtii*. We selected this latter plant model because (1) it is relatively simple to set up and resistant to nutritional limitation as may occur under standard NMR conditions that require high cell densities, (2) it is prone to easy modulation of its phosphorylated metabolites content in dark anaerobiosis conditions and/or after illumination and oxygen supply, and (3) our results can be put in perspective with earlier ^{31}P NMR studies on cultured green alga [15, 16].

2 Materials

Use commercial starting materials, reagents, and solvents without further purification. Prepare all solutions and buffers using doubly distilled deionized water (15 MΩ). Buffers and stock solutions of pH probes are to be used within 24 h following preparation. Store all phosphorylated pH probes and their precursors at –80 °C until use. Safety in handling chemicals and cell cultures and disposal of waste materials should comply with all regulations in place.

2.1 Chemical Analysis

Reactions were monitored by TLC on silica gel 60 aluminum plates with F254 as indicator (Merck). Flash chromatography purifications were performed on silica gel 60 (230–400 mesh). Analytical ^{1}H NMR (300.1 MHz), ^{13}C NMR (75.5 MHz), and ^{31}P NMR (121.5 MHz) spectra were recorded in $CDCl_3$ (Bruker AVL300 spectrometer). Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (^{1}H and ^{13}C) or external H_3PO_4 (^{31}P). Coupling constants (J) are reported in Hertz (Hz) and abbreviations of multiplicity were as follows: d, doublet; t, triplet; m, multiplet. Melting points were determined on a Stuart melting point apparatus SMP30 (Bibby Scientific, UK) and are not

corrected. HRMS were done on a Q-STAR Elite apparatus. All analytical experiments were performed at the analytical laboratory Spectropole located on the campus of Saint-Jérôme in Marseille, France.

2.2 Biology

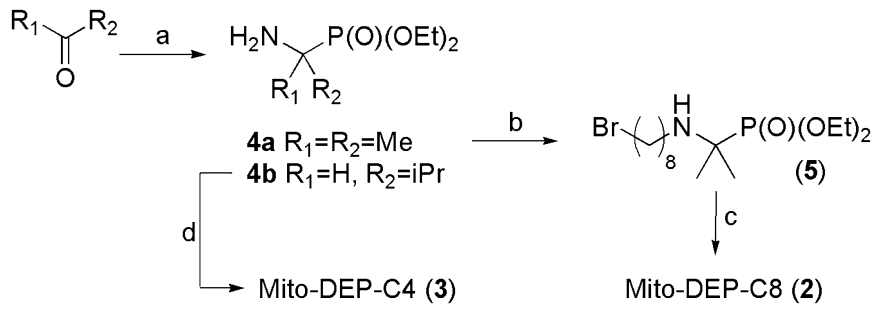
1. Tris-acetate-phosphate (TAP) salts stock solution (Beijerincks solution) [17]: Weigh 16.0 g NH_4Cl, 5.3 g $MgSO_4{\cdot}7H_2O$, 2.0 g $CaCl_2{\cdot}2H_2O$ and dissolve in 1 L water.
2. Trace elements solution (TES) [18]: To 850 mL H_2O, add 39.17 g EDTA·$2H_2O$ and 5 N KOH (107.2 mL). Once EDTA is completely dissolved add the following: 17.9 μM $FeCl_3{\cdot}6H_2O$, 76.5 μM $ZnSO_4{\cdot}7H_2O$, 6.3 μM $CuSO_4{\cdot}5H_2O$, 6.8 μM $CoCl_2{\cdot}6H_2O$, 25.6 μM $MnCl_2{\cdot}4H_2O$, 6.2 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 184 μM H_3BO_3. Do not adjust the pH which should be ~4.1. Make up to 1 L with water. The mixture should eventually turn purple. Filter the brown precipitate and store at +4 °C.
3. TAP medium: 20 mM Tris–HCl, TAP salts stock solution (a volume so as to reach a dilution 1/40 of the stock solution), 1 mM KH_2PO_4, TES (a volume so as to reach a dilution 1/1,000 of the stock solution), 18 mM glacial acetic acid and autoclave the solution, pH 7.05–7.20.
4. Alga cells (D66 strain obtained from the *Chlamydomonas* Resource Center (http://chlamycollection.org/)). Algal culture was observed on a biological microscope (Motic BA130, Germany). The mitochondrial respiration was followed on an oximeter (Hansatech Instruments, UK).
5. Stock solution of mito-DEP-C8 (**2**): 50 mM compound **2** (see synthetic procedure below) in TAP medium containing 0.5 % DMSO.

2.3 ^{31}P NMR Measurements

1. *Trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid monohydrate (CDTA) (Sigma). CDTA was used to maintain the concentration of divalent cations to very low values.
2. Bovine serum albumin (BSA).
3. 3-(*N*-morpholino)propanesulfonic acid (MOPS).
4. Saline intracellular protein-rich medium (ProtM) [12]: 40 mM KCl, 4 mM NaCl, 2.5 mM KH_2PO_4, 5 mM CDTA, 40 mg/mL BSA, pH 7.0. ProtM mimics the acidic vesicle environment.
5. Intramitochondrial-like buffer (MitoM) [19]: 140 mM KCl, 15 mM disodium succinate, 10 mM sodium glutamate, 20 mM MOPS, 1 mM CDTA, pH 7.0.
6. Cytosol-like buffer (CytoM) [10]: mince a freshly excised rat heart (~25 g, from the Wistar strain, CERJ Janvier, France) in 35 mL of 125 mM KCl at 2 °C, homogenize in a blender for 10 min, centrifuge at 10,000 × *g* for 15 min at 2–3 °C, and take the clear supernatant, pH 7.0. Store at –80 °C.

3 Methods

1. Synthesis of diethyl(2-methylpyrrolidin-2-yl)phosphonate (DEPMPH) (**1**) [9, 10]: Diethylphosphite (Sigma) (10.9 g, 79 mmol) and 2-methyl-1-pyrroline (Sigma) (7.2 g, 86 mmol, 1.1 eq.) were stirred at room temperature for 7 days. Water (50 mL) was then added and pH was adjusted to 2 with 37 % HCl. The aqueous layer was extracted with *tert*-butyl methyl ether (3 × 50 mL), its pH was adjusted to 10 with NaOH pellets, and the aqueous phase was extracted with dichloromethane (4 × 50 mL). The organic layers were gathered, dried over $MgSO_4$, filtered and concentrated. The residue was purified by distillation under reduced pressure to give DEPMPH as a colorless oil (14.8 g, 84 %). Bp 51 °C/4.5×10^{-7} mmHg; ^{1}H NMR ($CDCl_3$) δ (ppm) 4.17–3.97 (m, 4H, 2 × OCH_2), 3.04–2.88 (m, 2H, CH_2N), 2.22–2.10 (m, 1H, H-3), 1.82–1.45 (m, 4H, NH, H-3, 2 × H-4), 1.30–1.22 (m, 9H, 3 × CH_3); ^{31}P NMR ($CDCl_3$) δ (ppm) 30.85; ^{13}C NMR ($CDCl_3$) δ (ppm) 64.5 (d, J = 7.3 Hz, OCH_2), 64.4 (d, J = 7.3 Hz, OCH_2), 59.8 (d, J = 163.8 Hz, C-2), 47.2 (d, J = 7.3 Hz, C-5), 34.9 (d, J = 2.2 Hz, C-4), 25.7 (d, J = 5.3 Hz, C-3), 23.2 (d, J = 5.1 Hz, C–CH_3), 16.6 (d, J = 5.3 Hz, CH_2–CH_3).
2. Synthesis of diethyl 2-aminopropan-2-ylphosphonate (**4a**): Acetone (10 mL, 139 mmol) was reacted with dry ammonia at room temperature for 1 h. Diethylphosphite (17.8 mL, 139 mmol, 1 eq.) was then slowly added and the mixture was refluxed for 4 h. The solution was allowed to cool and then concentrated to dryness. The residue was distillated under reduced pressure to give **4a** (*see* structure in Scheme 1) as a colorless oil (10.5 g, 39 %). Bp 52–54 °C/3.0 mmHg; ^{1}H NMR ($CDCl_3$) δ (ppm) 4.12–4.01 (m, 4H, 2 × OCH_2), 1.30–1.16 (m, 12H, 4 × CH_3); ^{31}P NMR ($CDCl_3$) δ (ppm) 32.39; ^{13}C NMR ($CDCl_3$) δ (ppm) 62.3 and 62.2 (2 × OCH_2), 49.0 (d, J = 147.7 Hz, C), 25.1 (d, J = 3.8 Hz, CH_3), 16.6 and 16.5 (2 × CH_3); HRMS-ESI: calcd for $C_7H_{18}NO_3P$ ($[M+H]^+$) 196.1097, found 196.1085.
3. Synthesis of diethyl 2-(8-bromoactylamino)propan-2-ylphosphonate (**5**): To a solution of **4a** (917 mg, 4.70 mmol) in acetonitrile (20 mL) was added 1,8-dibromooctane (Sigma) (1.3 mL, 7.05 mmol, 1.5 eq.) over 10 min and the mixture was refluxed for 1 day. The mixture was allowed to cool and the precipitate was filtered. The filtrate was then concentrated to dryness and the residue was purified by flash chromatography (CH_2Cl_2/EtOH, 98/2, v/v) to give **5** (*see* structure in Scheme 1) as a yellow oil (0.87 g, 48 %). ^{1}H NMR ($CDCl_3$) δ (ppm) 4.12–4.01 (m, 4H, 2 × OCH_2), 3.31 (t, 2H, J = 6.8 Hz, CH_2Br), 2.62 (t, 2H, J = 6.8 Hz, CH_2), 1.80–1.70 (m, 2H,



Scheme 1 Synthesis of mito-targeted pH probes mito-DEP-C8 (**2**) and mito-DEP-C4 (**3**). Reagents and conditions: (**a**) (1) NH_3, RT, 1 h; (2) diethylphosphite, reflux, 4 h; (**b**) K_2CO_3, 1,8-dibromooctane, CH_3CN, reflux, 1 day; (**c**) PPh_3, CH_3CN, reflux, 3 days; (**d**) 4-iodobutyltriphenylphosphonium iodide, K_2CO_3, CH_3CN, reflux, 3 days

CH_2–CH_2Br), 1.41–1.15 (m, 22H); ^{31}P NMR ($CDCl_3$) δ (ppm) 31.84; ^{13}C NMR ($CDCl_3$) δ (ppm) 61.9 and 61.8 ($2\times OCH_2$), 53.2 (d, J=146.0 Hz, C), 42.7 (d, J=4.9 Hz, CH_2), 33.7 (CH_2), 32.5 (CH_2), 30.6 (CH_2), 29.0 (CH_2), 28.4 (CH_2), 27.8 (CH_2), 26.9 (CH_2), 23.7 (d, J=2.7 Hz, $2\times CH_3$), 16.4 and 16.3 ($2\times OCH_2$–CH_3); HRMS-ESI: calcd for $C_{15}H_{33}BrNO_3P$ ($[M+H]^+$) 386.1454, found 386.1450.

4. Synthesis of 8-(2-diethylphosphorylpropan-2-ylamino)-octyltriphenylphosphonium bromide (mito-DEP-C8) (**2**): Triphenylphosphine (500 mg, 1.91 mmol, 1.7 eq.) was added to a solution of **5** (431 mg, 1.12 mmol) in acetonitrile (15 mL) and the mixture was refluxed for 3 days under argon in the dark. The solution was allowed to cool and was concentrated to dryness under reduced pressure. The residue was dissolved in dichloromethane (10 mL) and the solution was added dropwise to vigorously stirred Et_2O (50 mL). The solvent was decanted and the yellow pale oil was dissolved in dichloromethane (5 mL) and the resulting solution was added dropwise to vigorously stirred Et_2O (30 mL). This procedure was repeated two more times and the resulting precipitate was dissolved in dichloromethane (20 mL). The solution was washed with 15 % aqueous NaBr, dried over $MgSO_4$, filtered, and concentrated to dryness. Compound **2** was obtained as a sticky yellow oil (0.18 g, 25 %). ^1H NMR ($CDCl_3$) δ (ppm) 7.90–7.60 (m, 15H, $3\times Ph$), 4.12–4.01 (m, 4H, $2\times OCH_2$), 3.70–3.55 (m, 2H, $CH_2P^+Ph_3$), 2.60 (t, 2H, J=6.8 Hz, CH_2), 1.65–1.50 (m, 4H), 1.37–1.10 (m, 20H); ^{31}P NMR ($CDCl_3$) δ (ppm) 31.51, 24.42; ^{13}C NMR ($CDCl_3$) δ (ppm) 134.9 (d, J=2.7 Hz, $-P^+Ph_3$ *para*), 133.4 (d, J=9.9 Hz, $-P^+Ph_3$ *ortho*), 130.4 (d, J=12.6 Hz, $-P^+Ph_3$ *meta*), 118.1 (d, J=85.6 Hz, $-P^+Ph_3$ *ipso*), 61.9 and 61.8 ($2\times OCH_2$), 53.1 (d, J=146.0 Hz, C), 42.7 (d, J=5.5 Hz, CH_2), 30.8 (CH_2), 30.2 (d, J=15.9 Hz, CH_2–CH_2–CH_2–P^+Ph_3), 28.9 (–$(CH_2)_2$–), 27.0 (CH_2), 22.8

(d, $J = 3.3$ Hz, $2 \times CH_3$), 22.5 (d, $J = 50.6$ Hz, CH_2-P^+Ph_3), 22.4 (d, $J = 3.7$ Hz, CH_2–CH_2–P^+Ph_3), 16.5 and 16.4 ($2 \times OCH_2$–CH_3); HRMS-ESI: calcd for $C_{33}H_{48}NO_3P_2^+$ 568.3104, found 568.3104.

5. Synthesis of diethyl 2-aminopropan-2-ylphosphonate (**4b**): Isobutyraldehyde (Sigma) (12.7 mL, 139 mmol) was reacted with dry ammonia at room temperature for 1 h. Diethylphosphite (17.8 mL, 139 mmol, 1 eq.) was then slowly added and the mixture was refluxed for 4 h. The solution was allowed to cool and then concentrated to dryness. The residue was distillated under reduced pressure to give **4b** (*see* structure in Scheme 1) as a colorless oil (10.0 g, 34 %). Bp 47–49 °C/3.1 mmHg; ^{1}H NMR ($CDCl_3$) δ (ppm) 4.12–4.01 (m, 4H, $2 \times OCH_2$), 2.78–2.74 (m, 1H), 2.09–1.97 (m, 1H, C*H*$(CH_3)_2$), 1.26 (t, 6H, $J = 7.0$ Hz, $2 \times OCH_2CH_3$), 0.98 (d, 3H, $J = 7.0$ Hz, CH_3), 0.94 (d, 3H, $J = 7.0$ Hz, CH_3); ^{31}P NMR ($CDCl_3$) δ (ppm) 28.61; ^{13}C NMR ($CDCl_3$) δ (ppm) 61.7 and 61.6 ($2 \times OCH_2$), 54.1 (d, $J = 146.0$ Hz, CH), 29.0 ($CH(CH_3)_2$), 20.5 (d, $J = 13.2$ Hz, CH_3), 17.2 (d, $J = 4.4$ Hz, CH_3), 16.4 and 16.3 ($2 \times OCH_2CH_3$); HRMS-ESI: calcd for $C_8H_{20}NO_3P$ ($[M+H]^+$) 210.1254, found 210.1261.

6. Synthesis of 4-(1-diethylphosphoryl-2-methylpropylamino)-butyltriphenylphosphonium bromide (mito-DEP-C4) (**3**): To a solution of **4b** (1.25 g, 6.00 mmol, 1.02 eq.), potassium carbonate (2.20 g, 15.9 mmol, 2.7 eq.) in acetonitrile (25 mL) was added 4-iodobutyltriphenylphosphonium iodide (Sigma) (3.37 g, 5.90 mmol) over 10 min. The mixture was refluxed for 3 days in the dark under argon and then allowed to cool. The precipitate was filtered and the filtrate was concentrated to dryness. The residue was dissolved in dichloromethane (10 mL) and the solution was added dropwise to vigorously stirred Et_2O (50 mL). The solvent was decanted and this dichloromethane-Et_2O extraction was repeated. After decantation, the residue was dissolved in water (10 mL); the solution was acidified with a 1 N aqueous HCl and extracted with ethylacetate (2×10 mL). The aqueous layer was basified with a 10 % aqueous NaOH and extracted with dichloromethane (2×20 mL). The organic layers were gathered, dried over $MgSO_4$, filtered, and concentrated to dryness to give **3** as a sticky yellow oil (2.5 g, 70 %). ^{1}H NMR ($CDCl_3$) δ (ppm) 7.90–7.60 (m, 15H, $3 \times Ph$), 4.08–4.01 (m, 4H, $2 \times OCH_2$), 3.81–3.69 (m, 2H, $CH_2P^+Ph_3$), 2.95–2.57 (m, 3H), 2.11–2.00 (m, 1H, C*H*$(CH_3)_2$), 1.89–1.70 (m, 4H), 1.26 (t, 6H, $J = 7.0$ Hz, OCH_2CH_3), 0.91 (m, 6H, $2 \times CH_3$); ^{31}P NMR ($CDCl_3$) δ (ppm) 28.45, 24.41; ^{13}C NMR ($CDCl_3$) δ (ppm) 135.0 (d, $J = 2.9$ Hz, $-P^+Ph_3$ *para*), 133.7 (d, $J = 10.2$ Hz, $-P^+Ph_3$ *ortho*), 130.5 (d, $J = 12.5$ Hz, $-P^+Ph_3$ *meta*), 118.2

(d, J = 85.8 Hz, –P^+Ph_3 *ipso*), 61.7 and 61.5 (2 × OCH_2), 60.4 (d, J = 143.0 Hz, CH), 48.4 (d, J = 2.9 Hz, CH_2), 30.4 (d, J = 16.9 Hz, CH_2–CH_2–CH_2–P^+Ph_3), 29.0 (d, J = 4.93 Hz, $CH(CH_3)_2$), 22.7 (d, J = 49.9 Hz, CH_2-P^+Ph_3), 20.8 (d, J = 13.2 Hz, CH_3), 20.1 (d, J = 49.9 Hz, CH_2–CH_2–P^+Ph_3), 17.8 (d, J = 3.7 Hz, CH_3), 16.6 and 16.5 (2 × OCH_2–CH_3); HRMS-ESI: calcd for $C_{30}H_{42}NO_3P^+_2$ 526.2634, found 526.2633.

7. Grow *C. reinhardtii* on TAP medium at 24 °C in light conditions (50 µE/m^2/s), under constant shaking (125 rpm) [20]. The cells were harvested in their log phase and then were resuspended in 1/5 vol of fresh TAP medium (pH 7.05) to reach cell densities of ~2–3 × 10^7 cells/mL. Cell suspension was transferred into an Erlenmeyer and kept under light and agitation until use.
8. Toxicity of mito-DEP-C8 on alga using the Evans blue dye method [21]: Cells were incubated for 1 h at 25 °C in darkness or in the presence of light in TAP medium supplemented with 0.1 mM or 0.5 mM of **2**. Equal volumes of algal cultures and 0.1 % Evans blue dye (Sigma), a reagent which penetrates into the algal cells and is expulsed from healthy cells, were then combined and incubated for 15 min at room temperature. An aliquot of this mixture was then transferred to a hemocytometer for cell counting. We found that both tested concentrations of **2** induced no cytotoxic effects since no blue coloration was observed and vitality of the alga was similar to that seen in control cells incubated in TAP medium containing 0.005 % DMSO.
9. Toxicity of mito-DEP-C8 on alga dark-aerobic respiration: Cells were grown exponentially, harvested by centrifugation (5,000 × *g* for 7 min) at room temperature, and resuspended in TAP medium (3–4 × 10^8 cells/mL). Aliquots of 200 µL of concentrated cell suspension were kept in darkness for 15 min in TAP medium alone or containing 0.1–1 mM of **2**. Aliquots (50 µL) of the incubation medium were injected in a Clark-type electrode cell that contained 1 mL of TAP medium at 24 °C and oxygen consumption was monitored in the dark for 3 min. The basal rate of O_2 consumption was of 16 ± 1.5 nmol O_2/min/mL. We found that compound **2** had a slight effect on the algal dark respiration at 1 mM, but not at 0.5 mM, with respiration rates of 10 ± 2 nmol O_2/min/mL and 14 ± 2 nmol O_2/min/mL, respectively.
10. Protocol of ^{31}P NMR titration: Dissolve the tested compound **1**–**3** (2 mM) in appropriate buffer (i.e., ProtM, MitoM, or CytoM) supplemented with 5 mM KH_2PO_4 (as a model for P_i). Adjust the pH to 10 to 20 different values in the range of 1.0–12.0 with HCl or NaOH solutions. Place each solution in

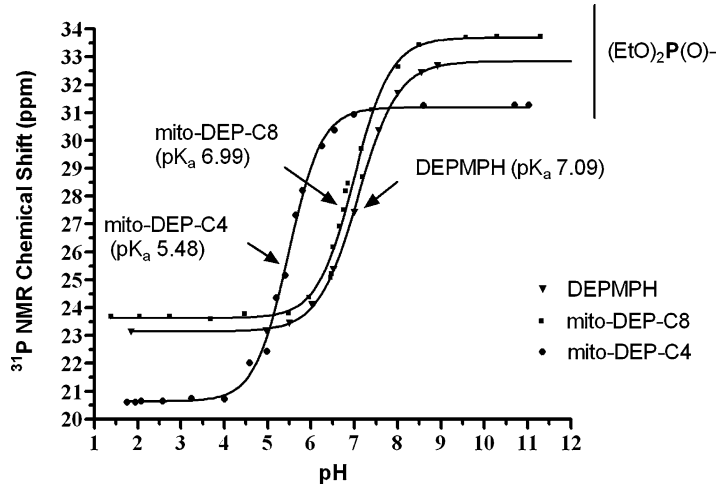


Fig. 2 ^{31}P NMR titration curves of pH probes DEPMPH (**1**) in CytoM medium, mito-DEP-C8 (**2**) in MitoM medium, and mito-DEP-C4 (**3**) in ProtM medium. Limiting ^{31}P NMR chemical shifts (in ppm) in acidic and basic medium (δ_A, δ_B): (**1**) 23.14, 32.83; (**2**) 23.62, 33.69; (**3**) 20.74, 31.27. Literature data for inorganic phosphate in Cyto-M medium: $\delta_A = -0.13$ ppm, $\delta_B = 2.45$ ppm, and $pK_a = 6.79$ (*see* ref. 10). For compounds **2** and **3**, only the (EtO)$_2$**P**(O)-group was considered for the pH determination (*see* **Note 1**)

a 10-mm NMR tube and record the ^{31}P NMR spectrum (here, we used a Bruker AMX 400 spectrometer operating at a phosphorus frequency of 161.98 MHz) at 25 °C using a small capillary filled with D_2O as a lock signal. For each test compound monophasic titration curves (Fig. 2) were obtained using the Henderson-Hasselbalch equation by iteratively fitting the δ versus pH values [9] (*see* **Note 1**). This allows to calculate the respective pK_a, δ_A, and δ_B values (GraphPad Prism software, USA).

11. Protocol of subcellular pH determination in living algal culture by ^{31}P NMR using **2** as the probe: Centrifuge 35 mL algal suspension in TAP at 6 °C (1,900 × *g* for 7 min). Resuspend in 0.2 mL TAP medium, pH 7.05 (to reach a density of ~4×10^9 cells/mL) and add 0.4 mL of TAP containing **2** (as to reach 179 μM). Transfer the mixture into a 5-mm NMR tube and record the ^{31}P NMR spectrum under D_2O lock conditions using a ZGIG sequence with a 30° (10.70 μs) pulse width, acquisition time of 0.67 s, repetition delay of 2.00 s, and sequential ~20-min data storage (i.e., 512 scans). A typical spectrum is shown in Fig. 3a (here, we used a Bruker Avance III spectrometer operating at a phosphorus frequency of 242.94 MHz) (*see* **Note 2**).

    Figure 3a illustrates the potential of the nontoxic **2** to simultaneously probe the cytosolic (i.e., 6.98, as compared to

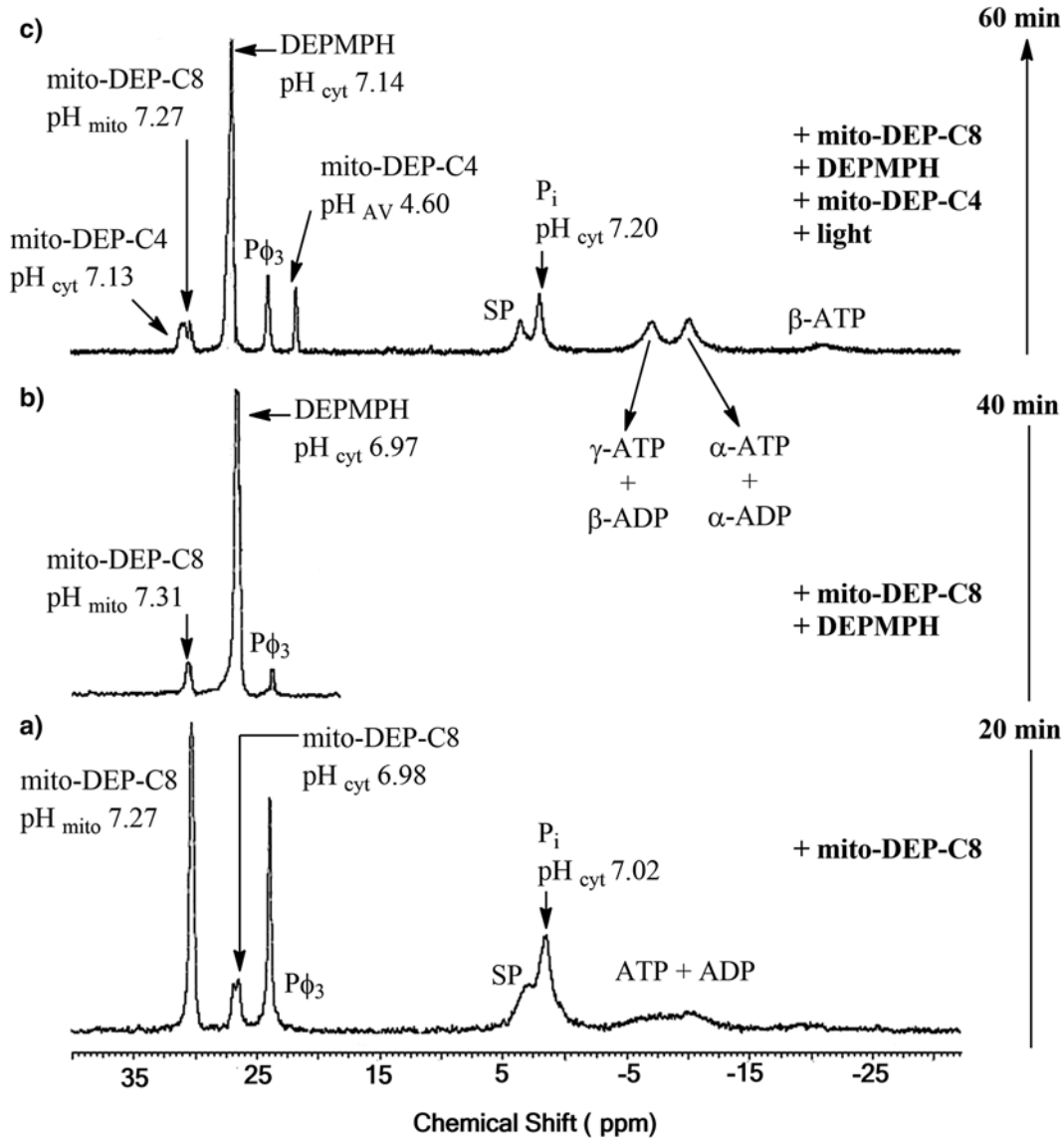


Fig. 3 Typical ^{31}P NMR spectra at 25 °C from D66 *C. reinhardtii* cells (4×10^9 cells/mL) in TAP buffer (600 μL) in the presence of pH probes: (**a**) under dark anaerobic conditions in the presence of 178 μM **2**; (**b**) under dark anaerobic conditions in the presence of a mixture of 178 μM **2** + 1.08 mM **1**; (**c**) same as (**b**) after addition of 214 μM **3** followed by illumination and air gassing for 2 min. The pH values of subcellular compartments were calculated from the titration curves of Fig. 2; determination of the cytosolic pH was made according to P_i titration. The chemical shift of the triphenylphosphonium cation $P\phi_3$ was constant at 23.95 ppm. *AV* acidic vesicles, *SP* sugar phosphates

the value of 7.02 given by the shift of the P_i peak) (*see* **Note 3**) and mitochondrial pH (i.e., 7.27) in living alga. This more alkaline value in the mitochondria is in agreement with the pH range ~6.5–8.0 determined by fluorescence techniques in plants or mammals [4–8].

12. Confirming the cytosolic pH determination in alga using a (**2** + **1**) mixture: Use the NMR tube containing the **2-supplemented** cell suspension in TAP medium as described above, add **1** as to obtain a final concentration of 1.08 mM, and record a new ^{31}P NMR spectrum using the above parameters (Fig. 3b).

    As expected from its lack of mitochondrial affinity, **1** only distributes within the cytosolic compartment, yielding a pH value of 6.97, in agreement with the value seen in Fig. 3a with **2**, the resonance peak of which is overlapped by that of **1** in this region (Fig. 3b). Compound **2** still efficiently probes the mitochondrial pH at 7.31.

13. Probing the acidic subcellular compartments in photosynthetically active alga by adding **3**: Use the NMR tube containing the (**2** + **1**)-supplemented cell suspension in TAP medium as described above, add **3** as to obtain a final concentration of 214 μM, illuminate (100 W bulb) and gas with air for 2 min, and record a new ^{31}P NMR spectrum using the above parameters (Fig. 3c).

    As expected from its lower pK_a of 5.48 (Fig. 2), **3** selectively probed the acidic vesicles as shown by its shielded peak corresponding to pH 4.60, but did not reach the mitochondria contrary to **2** which still probes this organelle, giving a pH of 7.27 (Fig. 3c). Interestingly **3** also probed the cytosol (giving pH 7.13 from its deshielded peak), a result in agreement with the information afforded by **1** (pH 7.14) and P_i (pH 7.20). Compared to the situation of Fig. 3a (i.e., for anaerobic and dark cells), this observed increase of cytosolic pH, concomitant with the rise of ATP and/or ADP levels (see the $-15 < \delta < -5$ ppm region), confirms that the photosynthetic activity of alga has been partially reactivated.

4 Notes

1. Compounds **2** and **3** bear two phosphorus atoms, yielding two ^{31}P NMR peaks. It has been demonstrated that the chemical shift associated with the triphenylphosphonium group remained constant at ~24 ppm in the 1.5–12 pH range. In the meantime, the chemical shift associated with the diethylphosphonate group was pH dependent (*see* ref. 14).
2. When algae are incubated in TAP medium, there is no nutritional limitation. The acetate contained in the medium is sufficient to enable the cell to live and grow in the absence of light for the duration of anaerobic NMR experiments (at least 40 min).

3. It is worth noting that whatever the medium (CytoM, ProtM, and MitoM) used for titration experiment P_i is added simultaneously (5 mM) to the pH probe (1–2 mM) in the mixture. Thus, characteristics of P_i titration (pK_a, δ_A and δ_B) were obtained in each medium. Furthermore, we have checked that ^{31}P NMR chemical shift of P_i is only pH and medium (composition in ions, proteins) dependent and invariant with the pH probe nature.

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