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In Vivo Effects of Photosynthesis Inhibitors in *Synechococcus* As Determined by ^{31}P NMR Spectroscopy†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance spectra were obtained from darkened cells of the unicellular cyanobacterium *Synechococcus* sp. Resonance peaks were assigned to intracellular pools of sugar–phosphates, inorganic phosphate (P_i), nucleotides, and polyphosphate. An internal pH of 7.2 was estimated from the chemical shift of the P_i resonance. Cells were then illuminated at 1600 $\mu E \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation by a fiber optic cable immersed in the cell sample. Spectra obtained after approximately 15 min of illumination showed an increase in nucleotide pools and an increase in the cytoplasmic pH to 7.6. In the presence of 0.3 mM dinitrophenol (DNP), an uncoupler of phosphorylation, spectra of illuminated cells showed an immediate decline in nucleotide pools while sugar–phosphate levels remained constant. Addition of the photosystem II (PS II) electron-transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (7.2 μM) did not affect nucleotide levels in the cells during the time course of the experiment (15–30 min). However, an abrupt rise in the resonance in the sugar–phosphate region was noted. Spectra of DCMU-treated cell extracts indicated that one metabolite was principally responsible for the change in pool size. The metabolite was identified as 3-phosphoglyceric acid. Spectra of illuminated cells were also obtained in the presence of the natural herbicide cyanobacterin. Cyanobacterin inhibits electron transport in PS II. The mechanism by which the inhibitor acts is unknown. Unlike results obtained with DNP or DCMU, spectra of cyanobacterin-treated cells showed no major changes in nucleotide or sugar–phosphate resonances. A slow decline in cytoplasmic pH was seen in the presence of cyanobacterin, indicating that the natural product affects the proton pumping mechanism in PS II.

Recently, whole cell and tissue nuclear magnetic resonance techniques have been used to study various metabolic processes in plants. For example, intracellular pH changes in cytoplasm and vacuoles have been estimated in maize (Roberts et al., 1980) and other tissues grown in culture (Martin et al., 1982). In addition, the concentrations of various phosphorylated metabolites in plant cells have been estimated by ^{31}P NMR (Mitsumori et al., 1985). Other nuclei such as ^{13}C and ^{15}N have also been used as tracers in studies in plant metabolism [see Roberts (1984) for a review].

However, the major physiological process which occurs in plants is photosynthesis. NMR could be used to study in vivo pH and metabolite changes in plant tissues after the dark to light transition. The method should be useful in confirming previous in vitro studies and could be extended to the investigation of metabolite pools and herbicide activity during photosynthetic electron transport and carbon dioxide fixation. A few reports on photosynthesis using NMR methods have been published, but several technical problems limit the usefulness of the technique. Since NMR is relatively insensitive, thick cell suspensions are required to obtain reproducible signals. Providing enough light and carbon dioxide to cells

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in the spectrometer then become major problems. We have overcome this difficulty by using a fiber optic system immersed in the cell sample and lowered into the spectrometer. Our method is a simplification of a similar device described previously (Mitsumori & Ito, 1984b). Our cells were mixed and provided with CO_2 through a capillary bubbling system which was successfully used for bacterial suspensions (Ugurbil et al., 1982).

Another problem in utilizing ^{31}P NMR on cells in general, and plant cells in particular, is the assignment of inorganic and metabolite phosphorus resonances to compounds in different intracellular compartments. In only a few tissues has this been possible (Ugurbil et al., 1979, 1984). For our studies, we used the simplest oxygen-evolving photosynthetic microorganisms, the cyanobacteria. These organisms lack intracellular compartments except for the intrathylakoid space. However, estimates of the size of this space suggest that it will not contribute to NMR spectra. A previous report has confirmed this suggestion (Kallas & Dahlquist, 1981). The cyanobacteria have a photosynthesis system and carbon dioxide fixation cycle comparable to that found in chloroplasts of higher plants. In addition, they are easy to grow in large quantities in the laboratory. With the proper strain, it is possible to obtain thick, homogeneous suspensions which remain metabolically active for several hours under NMR conditions. Utilizing such an organism, *Synechococcus* sp. (ATCC 27146), we have investigated the effects of inhibitors on pH and phosphorylated metabolites during photosynthesis. We have used the classic uncoupler 2,4-dinitrophenol and the photosynthetic electron-transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The results of the NMR analysis confirm in vitro biochemical investigations. We have extended our studies to the activity of the natural product cyanobacterin, which inhibits electron transport at an unknown site in photosystem II (PS II)[1] (Gleason & Paulson, 1984). The observed in vivo activity of cyanobacterin was quite different from that described for the known inhibitors and indicates a unique site of action for the natural product.

Materials and Methods

Cyanobacterial Culture Conditions. *Synechococcus* sp. (ATCC 27146) was maintained on liquid Bg 11 medium (Stanier et al., 1971) at room temperature and constant illumination. To obtain large quantities of cells, the cyanobacteria were grown on the same medium supplemented with 1 g of Na_2CO_3/L. Three hundred milliliters of exponential phase culture was used to inoculate a 10-L carboy containing 9 L of the above medium. The final pH was 7.6. The cultures were grown at 30 °C and a constant illumination of 670 $\mu E \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation. Cell growth was determined by direct counting in a Petroff–Hausser counting chamber. Cells were harvested approximately 2 days after inoculation when in the early exponential to mid-exponential growth phase.

Preparation of Samples. Cells were harvested by using a Millipore Pellicon cassette system with a 0.45-μm membrane. Cells were further concentrated by centrifugation at 2500*g* in a Sorvall RC-5B centrifuge. The cells were washed once in buffer containing 200 mM HEPES, pH 7.6, and 1 mM EDTA in Bg 11 medium without phosphate. The cells were resuspended in the same medium for NMR experiments. The final volume was 12 mL. The cell density in most experiments was approximately 2.0×10^{11} cells/cm^3. Chlorophyll was estimated after extraction with 90% acetone (Arnon et al., 1974). The chlorophyll concentration in the NMR samples was 2.3–3.0 mg/cm^3.

NMR Measurements. NMR spectra of the cyanobacteria were obtained on a Nicollet 360-MHz wide-bore instrument operating at a phosphorus frequency of 146.1 MHz. ^{31}P spectra were the Fourier transform of 800 free induction decays (FID) recorded with 80° pulses with a 0.4-s delay between pulses. The total time for the accumulation of each spectrum was 5.1 min. At exponential filter of 20 Hz was applied before Fourier transformation. Illumination of the sample was provided by an American-Optical 150-W lamp (Model 1185B) connected to a fiber optic cable. A silicone stopper was placed on the end of the cable to dissipate the heat generated by the lamp. A solid glass bulb was connected to the stopper to distribute the light, and the bulb was immersed in the algal sample in the NMR tube. The illumination as measured at the end of the bulb was 1600 $\mu E \cdot m^{-2} \cdot s^{-1}$. The sample was gently aerated with a mixture of 5% CO_2 in air through a three-capillary bubbling system (Ugurbil et al., 1982).

Preparation of Cell Extracts. Cells were removed from the spectrometer and immediately frozen in liquid nitrogen. The cyanobacterial samples were subsequently thawed, and distilled water was added to obtain a 20% cell slurry. The cells were disrupted by sonication for 20 min in a Heat Systems cell disruptor operating at 50% maximum power. The lysate was centrifuged at 5000*g* for 10 min and the supernatant extracted with 10% perchloric acid (v/v). Precipitated protein was removed by centrifugation as described above. The pellet was washed with 5% PCA, and the supernatants were pooled. The PCA was titrated with 1 N KOH to pH 8.0. The resulting precipitate was removed by filtration. The pH of the extract was adjusted to 7.0 with HCl, and the sample was lyophilized. The dried sample was resuspended in 80% D_2O buffered with 50 mM HEPES, pH 7.8, plus 50 mM EDTA. Samples were extracted at 4 °C and stored as dry powders at 4 °C or in frozen solution until spectra were taken.

^{31}P spectra of cell extracts were obtained on a Bruker 275-MHz narrow-bore instrument operating at 102.5 MHz. Spectra were proton decoupled with the sample maintained at 37 °C. An exponential filter of 5 Hz was applied before Fourier transformation.

2,4-Dinitrophenol (DNP), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and 3-phosphoglyceric acid (PGA) were obtained from Sigma Chemical Co., St. Louis, MO. Cyanobacterin was extracted from *Scytonema hofmanni* and purified as described elsewhere (Pignatello et al., 1983). Inhibitors were dissolved in anhydrous ethyl ether before addition to cell samples.

Results

The spectrum shown in Figure 1 is representative of the phosphorus metabolites which can be detected in *Synechococcus* cells after approximately 30 min of illumination. Nucleotide pools are quite evident at −5 ppm (γ-NTP, β-NDP), −10 ppm (α-NDP), and −19 ppm (β-NTP). A small polyphosphate pool is seen at −22 ppm. The phosphorylated sugars appear as a shoulder at approximately 3–5 ppm. In this spectrum, the inorganic phosphate peak is split due to the presence of P_i in the suspension medium. The P_i peak at 2.7 ppm (pH 7.4) is representative of the external medium which in this particular case was buffered with carbonate. The peak

[1] Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP, 2,4-dinitrophenol; NDP, nucleotide diphosphate(s); NTP, nucleotide triphosphate(s); PGA, 3-phosphoglyceric acid; P_i, inorganic phosphate(s); PS I, photosystem I; PS II, photosystem II; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PCA, perchloric acid; kDa, kilodalton(s).

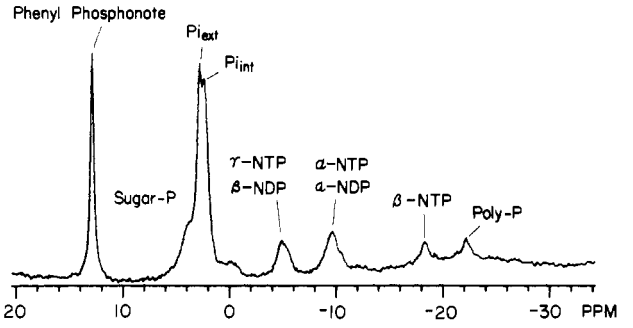

FIGURE 1: ^{31}P NMR spectrum of *Synechococcus* cells suspended in 0.24 M $NaHCO_3$ and 0.002 M EDTA prepared in complete Bg 11 medium (0.2 mM phosphate), pH 7.4. 2 mM phenylphosphonate was added to the suspension. The spectrum represents the accumulation of 800 pulses over 5.1 min. The chemical shift of phenylphosphonate at 12.8 ppm was used as an internal reference. Phenylphosphonate did not penetrate into *Synechococcus* cells. The P_i peak is split; the resonance at 2.7 ppm is due to external P_i at pH 7.4 while the resonance at 2.4 ppm is cytoplasmic P_i at a pH of 7.1. Other major resonances are assigned as follows: sugar–phosphate pool, shoulder at 5–3 ppm; γ-NTP, β-NDP, approximately −5 ppm; α-NTP, α-NDP, −10 to −12 ppm; β-NTP, −18 ppm; polyphosphate, −22 ppm.

at 2.4 ppm (pH 7.1) is cytoplasmic P_i. The standard, phenylphosphonate (12.8 ppm), was added to the algal suspension. Unlike previously reported results with *Escherichia coli* cells (Thoma et al., 1986), phenylphosphonate does not penetrate *Synechococcus* cells, and thus, the compound could be used as an extracellular reference only. In separate experiments utilizing an oxygen electrode, phenylphosphonate was shown to have no effect on photosynthetic electron transport in these cells, further confirming its inability to penetrate the *Synechococcus* cell membrane (data not shown).

Although we obtained satisfactory results with sodium carbonate buffering, the external pH was not constant in this medium, presumably because the cells are able to take up and utilize carbonate as an alternate CO_2 source. The subsequent decline in pH greatly decreased cell viability under NMR conditions. All further experiments were done in 200 mM HEPES buffer, pH 7.6, in the absence of added P_i as described above. The spectra of cells maintained in this medium are shown in Figure 2. In spectrum a, cells were harvested and placed in the spectrometer without illumination. The sugar–phosphate pool is noted at 4 ppm, but nucleotide pools are barely discernible under these conditions. The single P_i resonance at pH 7.2 is from internal phosphorus only. The spectrum shown in Figure 2b was obtained from the same cell sample after approximately 15 min of illumination. Nucleotide pools are now easily detected and the somewhat broader P_i peak caused by the shifting of the internal pH to 7.6 during acquisition of the data. The increase in nucleotide pools and the pH shift observed were presumably caused by light-driven electron transport. The classical uncoupler 2,4-dinitrophenol inhibits ATP synthesis while allowing electron transport to continue. Addition of 0.3 mM DNP to the cells in spectrum b causes an abrupt decline in nucleotide pools in *Synechococcus* as illustrated in Figure 2c. No obvious change in the sugar–phosphate metabolites is noted, and the internal pH, as determined from the chemical shift of P_i, remains at 7.6.

In contrast, addition of the known photosynthetic electron-transport inhibitor DCMU to illuminated cells does not result in a decline of the nucleotide pools (data not shown). The partial spectrum shown in Figure 3 illustrates an obvious shift in the sugar–phosphate metabolites after treatment with 7.2 μM DCMU. To identify the metabolite(s) which increase(s) upon treatment with the inhibitor, cell extracts were

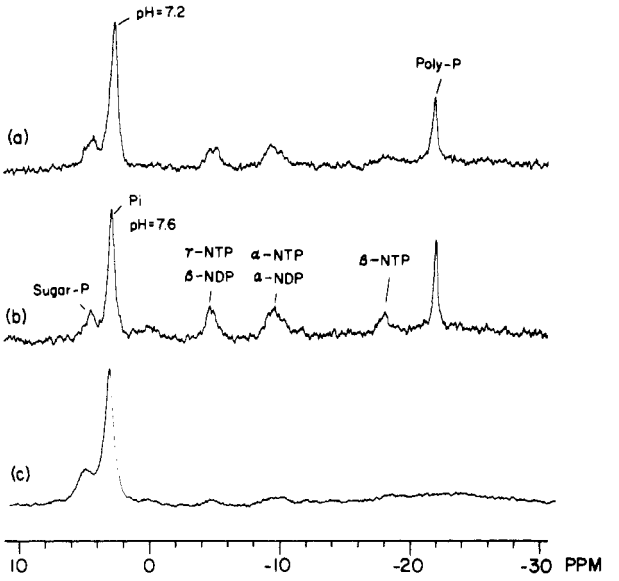

FIGURE 2: Effect of the dark to light transition and dinitrophenol on ^{31}P spectra of *Synechococcus*. (a) Cells were harvested and placed directly in the spectrometer without illumination. Cells were suspended in 200 mM HEPES, pH 7.6, and 2 mM EDTA in Bg 11 without phosphate. The cells were bubbled with a mixture of 5% CO_2 in air. Data accumulation and spectral assignments are as in Figure 1 except for P_i. The P_i resonance represents cytoplasmic phosphate at pH 7.2. The phenylphosphonate resonance is downfield of the region shown. (b) The cells in (a) were bubbled with 5% CO_2 in air and illuminated by a "light bulb" immersed in the sample which supplied 1600 $\mu E \cdot m^{-2} \cdot s^{-1}$ radiation as determined by a light meter placed at the end of the bulb. Data accumulation is as described in Figure 1. Resonance assignment is as above except for a P_i shift to 3.0 ppm (pH 7.6). (c) 0.3 mM dinitrophenol (final concentration) was added to the cells in (b). Data accumulation and resonance assignments are as above.

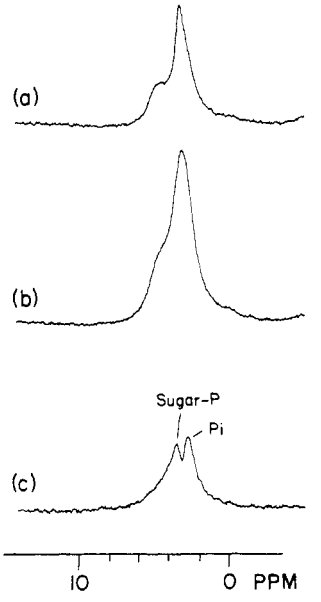

FIGURE 3: ^{31}P spectra of sugar–phosphate region in *Synechococcus* cells. (a) Spectrum of illuminated cell suspension. Cells were placed in the spectrometer and illuminated immediately as described in Figure 2b. Resonance assignment is 5–3 ppm for the sugar–phosphate pool and 3.0 ppm for P_i. (b) Spectrum after addition of 7.2 μM DCMU to the cell suspension shown in (a). Resonance assignment is as above. (c) Difference spectrum of (a) minus (b). The increased sugar–phosphate resonance is seen at approximately 3.7 ppm.

made of illuminated cells and cells treated with DCMU. Spectra of extracts are shown in Figure 4. An additional resonance at 4.55 ppm appears after treatment with DCMU (see Figure 4b). This chemical shift corresponds to that of

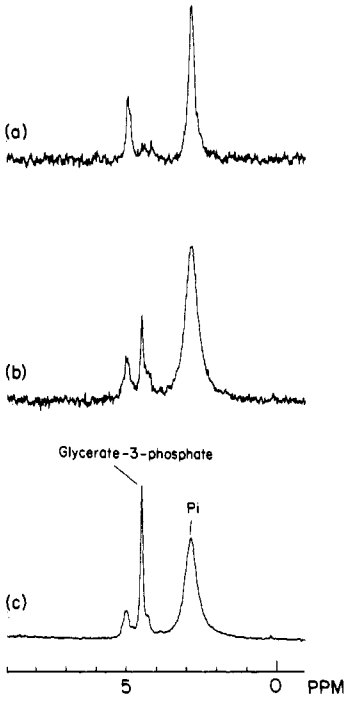

FIGURE 4: ^{31}P spectra of cell extracts. In a parallel experiment to that of Figure 3, *Synechococcus* cell suspensions were frozen in liquid nitrogen after illumination for 15 min and after illumination in the presence of 7.2 μM DCMU for 15 min. The frozen cells were thawed and extracted with perchloric acid. (a) Spectrum of extract of illuminated cells. The P_i resonance is at 2.9 ppm. The data are the result of the accumulation of 2000 scans. (b) Spectrum of extract of cells illuminated in the presence of 7.2 μM DCMU. The P_i resonance is at 2.9 ppm; the unknown metabolite resonance is at 4.55 ppm. The data are the result of the accumulation of 1000 scans. (c) Spectrum of (b) plus approximately 0.1 mM 3-phosphoglyceric acid. The data are the result of the accumulation of 10000 scans.

phosphoglyceric acid (Ugurbil et al., 1978). The metabolite in our extracts was identified as 3-PGA by adding authentic PGA to our sample. The spectrum in Figure 4c shows the additional PGA superimposed on the original resonance.

The natural product, cyanobacterin, also acts as an inhibitor of photosynthetic electron transport in PS II (Gleason & Paulson, 1984). However, the results of adding 7.8 μM cyanobacterin to illuminated cells are clearly distinguishable from those obtained with DNP or DCMU. As seen in Figure 5, addition of the natural product caused no major changes in nucleotide or sugar–phosphate pools. A shift of the P_i peak indicated a change in the internal pH. In the presence of cyanobacterin, the internal pH declined to 7.3, almost the level for dark cells, although the cells were continuously illuminated. Increasing the concentration of cyanobacterin had no effect on the resulting spectra.

Discussion

The metabolites detected in our ^{31}P NMR spectra with *Synechococcus* sp. are comparable to those reported by Kallas and Dalhquist (1981) using a thermophilic strain of the same organism. As expected, NMR is a relatively insensitive technique, and only the major phosphorylated metabolites (concentration greater than 0.1 mM) can be detected. However, the use of a more powerful instrument and a fiber optic lighting technique enabled us to significantly improve the signal to noise ratio over that of previous studies (Kallas & Dahlquist, 1981; Mitsumori & Ito, 1984a). Our technical improvements also made it possible to obtain cell spectra in 15–30 min as opposed to the 1–2 h previously required. Therefore, we were able to study the effects of light and inhibitors on the same

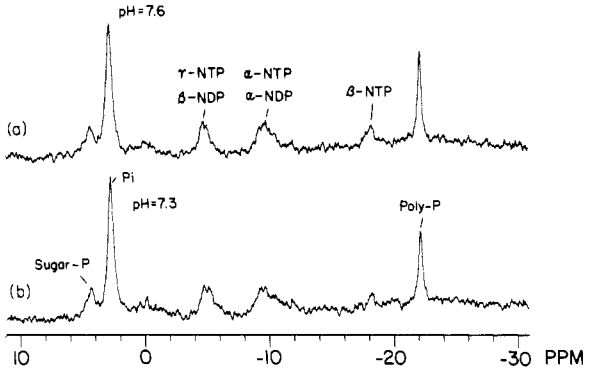

FIGURE 5: ^{31}P NMR spectra of *Synechococcus* cells before and after treatment with cyanobacterin. (a) Spectrum of illuminated cell suspension under conditions described in Figure 2b. Data collection and resonance assignments are as described in Figure 2b. (b) Spectrum of illuminated cells in (a) 15 min after addition of 7.8 μM cyanobacterin. The P_i resonance has shifted to 2.5 ppm after addition of the inhibitor, indicating a final pH of 7.3.

cell samples while in the spectrometer and determine initial changes in pH and phosphorus metabolism in vivo.

The spectra shown in Figure 2 are representative of metabolic events which can be observed on the dark to light transition. Cyanobacteria are relatively quiescent in the dark; rates of oxidative phosphorylation have been estimated at approximately 10% those of photosynthetic phosphorylation (Bottomley & Stewart, 1976). However, nucleotide levels are maintained by catabolism of glucose, principally by way of the pentose phosphate cycle (Smith, 1982). A moderate rise in ATP levels occurs on illumination as reflected in the somewhat higher nucleotide resonances seen in Figure 2b. There is no obvious change in the sugar–phosphate pools, presumably because similar intermediates are utilized in the pentose phosphate cycle as in the light-driven fixation of CO_2 (Calvin cycle). Subtle changes such as shifts in the relative amounts of ribulose bisphosphate and 3-phosphoglyceric acid in the light to dark transition (Pelroy et al., 1976) cannot be detected with the present technique. A shift in the P_i resonance on illumination is attributed to the light-driven pumping of protons out of the cytoplasm into the intrathylakoid space (Padan & Schuldiner, 1978). Under our conditions, we observe a rise in cytoplasmic pH of 0.4 unit which is similar to that previously reported (Kallas & Dahlquist, 1981). Measurements made of pH shifts in chloroplast stroma using non-NMR techniques (e.g., dimethyloxazolidinedione and methylamine; Heldt et al., 1973) suggest that a rise of 1 pH unit is typical. However, chemical techniques assume a free penetration of the pH dyes and no interaction with membrane components or metabolites [see, for example, Pfister and Homann (1986)]. In addition, use of any invasive technique cannot duplicate ideal in vivo conditions. Alternatively, the cells under NMR conditions may have been light-limited. The intensity of our NMR "light bulb" was approximately twice that under which the cells were grown and 10-fold higher than that required to maintain moderate rates of photosynthesis as determined with an oxygen electrode (Gleason & Paulson, 1984). As indicated by the rise in nucleotide pools, the conditions in the spectrometer were adequate to support photosynthesis and cell viability in the cyanobacteria over a period of several hours. Given the obvious buffering capacity of cyanobacterial cytoplasm and the ability of these organisms to maintain an alkaline internal pH (Kallas & Castenholz, 1982), a shift of 0.4 pH unit in the light may be optimal. A shift of similar magnitude has been reported in *Chlorella* chloroplasts by ^{31}P NMR (Mitsumori & Ito, 1984a).

Addition of the phosphorylation uncoupler 2,4-dinitrophenol causes an immediate decline in nucleotide levels as seen in Figure 2c. DNP is known to inhibit ATP synthesis and has also been reported to stimulate ATPase activity in mammalian mitochondria (Penefsky et al., 1960). A similar ATPase action may occur in the cyanobacteria since the decline in nucleotide pools occurs in the approximately 15 min needed to manipulate the sample and collect data. No obvious changes are seen in the phosphorylated sugar levels. Presumably, the cells can still reduce NADP and continue to fix CO_2 during this relatively short time period. In addition, no change is noted in the internal pH after DNP treatment. DNP presumably acts by discharging the transthylakoid pH gradient which should lead to a decline in the cytoplasmic pH. However, the uncoupler is also known to act on proton pumps located in the cytoplasmic membrane. It has been demonstrated that cyanobacteria are able to maintain an alkaline internal pH by an active-transport process and that the gradient can be extinguished by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (Kallas & Castenholz, 1982). In addition, a Ca^{2+}-ATPase antiport system has been described in the cyanobacterium *Anabaena variabilis* (Lockau & Pfeffer, 1983). Thus, the final internal pH of 7.6 after DNP treatment of *Synechococcus* is most likely a result of equilibration with the external medium which was buffered at a pH of 7.6.

The site of action of the photosynthetic electron-transport inhibitor DCMU has been well characterized. DCMU is known to bind to a 32-kDa quinone binding protein on the reducing side of PS II and prevent electron transfer from a primary quinone acceptor (Vermaas et al., 1984). DCMU, at low concentrations, has no effect on PS I. The levels of inhibitors we used were determined from previous experiments with the same organism and an oxygen electrode (Gleason & Paulson, 1984). The minimum inhibitory levels were then extrapolated to the cell densities used in the NMR spectrometer. In the presence of low concentrations of DCMU, *Synechococcus* should be able to continue cyclic photophosphorylation (Izawa, 1968), and thus, nucleotide levels are expected to remain at light levels during the time period of these experiments. However, as noted in Figure 3, there is an obvious rise in the amount of PGA. When noncyclic electron transport is inhibited by DCMU, cells can no longer reduce sufficient amounts of NADP to fix CO_2 which results in an accumulation of this initial product of the Calvin cycle.

Cyanobacterin is an allelopathic natural product isolated from the cyanobacterium *Scytonema hofmanni* (Mason et al., 1982). Like DCMU, it is also known to inhibit photosynthetic electron transport in PS II (Gleason & Paulson, 1984). However, a DCMU-resistant mutant of the cyanobacterium *Anacystis nidulans* was shown to be sensitive to cyanobacterin, indicating that the natural product does not act at the same site as the classical PS II inhibitors. Results using the Hill electron acceptor, silicomolybdic acid, suggest that cyanobacterin acts at a site on the oxidizing side of the primary quinone acceptor (Gleason et al., 1986). Cyanobacterin does not inhibit electron transport in PS I (Gleason & Case, 1986); thus, cells are expected to maintain nucleotide pools at light levels by cyclic photophosphorylation as shown in Figure 5b. However, in contrast to DCMU inhibition, no obvious change is seen in the phosphorylated sugar metabolites. Perhaps some residual NADP reduction is possible at the levels of cyanobacterin used in these experiments. Conversely, an alternate mechanism of reducing ferredoxin may operate in the presence of this inhibitor as has been reported for the plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (Arnon et al., 1981). The only change noted in vivo after cyanobacterin treatment is a slow decline in the cytoplasmic pH. The results suggest that in addition to inhibiting electron transport, cyanobacterin disrupts thylakoid membrane structure, allowing protons to slowly leak out of the thylakoid lumen. Unlike DNP, the natural product does not affect the cytoplasmic membrane. The NMR data corroborate previous evidence of a subtle effect of cyanobacterin on thylakoid membrane ultrastructure in the cyanobacteria. In addition, the natural product, unlike DCMU and related inhibitors (deVitry & Diner, 1984), does not affect organisms such as *Rhodospirillum rubrum* which lack an O_2-evolving photosynthetic system (Gleason & Paulson, 1984). We conclude that cyanobacterin acts at a site unique to PS II presumably close to the primary electon acceptor.

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Registry No. 3-PGA, 820-11-1; DNP, 51-28-5; DCMU, 330-54-1; PO_4^{3-}, 14265-44-2; cyanobacterin, 80902-00-7.

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Cloning and Sequence Analysis of cDNAs Encoding Mammalian Mitochondrial Malate Dehydrogenase†

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ABSTRACT: A cDNA clone, named ppmMDH-1 and covering a part of the porcine mitochondrial malate dehydrogenase (mMDH; L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) mRNA, was isolated from a porcine liver cDNA library with a mixture of 24 oligodeoxyribonucleotides as a probe. The sequences of the probe were deduced from the known sequence of porcine mMDH amino acid residues 288–293. ppmMDH-1 covered the coding region for porcine mMDH amino acid residues 17–314 and the 3′ untranslated region. Subsequently, mouse mMDH cDNA clones were isolated from a mouse liver cDNA library with the ppmMDH-1 cDNA as a probe. One of the clones, named pmmMDH-1 and containing a cDNA insert of about 1350 base pairs, was selected for sequence analysis, and the primary structure of the mouse precursor form of mMDH (pre-mMDH) was deduced from its cDNA sequence. The sequenced coding regions for the porcine and mouse mMDH mRNAs showed about 85% homology. When the deduced amino acid sequence of the mouse pre-mMDH was compared with that of the porcine mMDH, they shared a 95% homology, and the mouse pre-mMDH yielded a leader sequence consisting of 24 amino acid residues and a mature mMDH, consisting of 314 amino acid residues. The leader sequence contained three basic amino acid residues, no acidic residues, and no hydrophobic amino acid stretch. The mouse mMDH leader sequence was compared with those of three other rodent mitochondrial matrix proteins.

Malate dehydrogenase (MDH;[1] L-malate:NAD^+ oxidoreductase, EC 1.1.1.37), an NAD^+-dependent dehydrogenase, occurs in two isozymic forms in animal tissues, one in the cytosol and the other in the mitochondria (Edwards & Hopkinson, 1977; Delbrück et al., 1959). These isoenzymes are composed of about 300 amino acid residues. The amino acid sequence has been available only for porcine heart mitochondrial MDH (mMDH) (Birktoft et al., 1982). The two isoenzymes, in cooperation with aspartate aminotransferase (AspAT) isoenzymes, seem to play a pivotal role in the malate–aspartate shuttle operative in a metabolic coordination between cytosol and mitochondria, in various mammalian tissues (Williamson et al., 1973).

We initiated studies on the molecular basis of the regulation of cellular levels of mitochondrial and cytosolic isoenzymes in the malate–aspartate shuttle. The mouse seems to be the most suitable species for such studies. We recently isolated mouse mitochondrial AspAT (mAspAT) and cytosolic AspAT (cAspAT) cDNAs, using the cloned porcine mAspAT and cAspAT cDNAs as probes (Joh et al., 1985; Obaru et al., 1986). In ongoing work on this project, we attempted to isolate mouse mMDH cDNAs. Because there is no available amino acid sequence data for the mouse mMDH, we first isolated porcine mMDH cDNAs, and subsequently, using one of the cloned porcine mMDH cDNAs as a probe, we isolated mouse mMDH cDNAs.

Mouse mMDH is encoded by a nuclear gene assigned to chromosome 5 (Roderick & Davisson, 1984) and represents one of the typical enzymes synthesized as larger precursors on free ribosomes in the cytosol, transported through the

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[1] Abbreviations: MDH, malate dehydrogenase; mMDH, mitochondrial MDH; pre-mMDH, precursor form of mMDH; cMDH, cytosolic MDH; AspAT, aspartate aminotransferase; mAspAT, mitochondrial AspAT; pre-mAspAT, precursor form of mAspAT; cAspAT, cytosolic AspAT; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; ppmMDH, plasmid carrying porcine mMDH cDNA; pmmMDH, plasmid carrying mouse mMDH cDNA; kb, kilobase pairs; bp, base pairs.

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