APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1997, p. 347–350 Vol. 63, No. 1
0099-2240/97/$04.00+0
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Characterization of Di-*myo*-Inositol-1,1′-Phosphate in the Hyperthermophilic Bacterium *Thermotoga maritima*

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Received 12 July 1996/Accepted 11 October 1996

Di-*myo*-inositol-1,1′-phosphate (DIP) is present at a significant concentration (~160 nmol/mg of protein) in the cytoplasm of the hyperthermophilic bacterium *Thermotoga maritima*. The concentration of DIP was independent of the pH of the growth medium or the cell growth phase but increased with increasing concentrations of NaCl in the growth medium, reaching a maximum (~450 nmol/mg of protein) at 0.4 to 0.6 M NaCl. A large-scale purification procedure for DIP which yields approximately 18 g of DIP per kg of cells (wet weight) is described. Purified DIP was stable at 90°C for at least 5 h. The presence of DIP (50 mM) did not increase the stability at 90°C of pure forms of the hydrogenase or pyruvate ferredoxin oxidoreductase of *T. maritima*, suggesting that DIP is not a general thermoprotectant.

Hyperthermophilic microorganisms exhibit temperature optima for growth between 80 and 105°C and have been isolated from various volcanic environments (1, 5). Virtually all of them are classified within the domain *Archaea* (25), with the exception of species of *Thermotoga* (13) and *Aquifex* (14), which are placed in the domain *Bacteria*. An intriguing question is how hyperthermophiles stabilize various molecules, particularly proteins, at such extreme temperatures. Any significant insight into this problem obviously has many biotechnological ramifications. While most of the enzymes that have been purified from hyperthermophiles exhibit maximal catalytic activity above 90°C in vitro (1, 2), the assays typically used are of short duration, and many of the pure proteins are not stable over prolonged periods at the optimum growth temperature of the organism from which they were obtained (4, 15, 21). This observation has led to the notion that these organisms may contain solutes that play a general "thermoprotectant" role to stabilize some, if not all, of their cytoplasmic proteins. For example, the heterotrophic archaeon *Pyrococcus woesei* (optimal growth temperature [T_{opt}], 100°C) maintains an intracellular potassium ion concentration near 0.6 M (21), and potassium ions have been shown to increase the thermal stability and catalytic efficiency of an enzyme from the methanogen *Methanopyrus kandleri* (T_{opt}, 98°C) (7). However, this appears not to be a general phenomenon as some hyperthermophiles, such as *Thermoproteus tenax* (T_{opt}, 88°C), have intracellular concentrations of potassium (and sodium) ions below 0.1 M (12).

The cytoplasm of *P. woesei* also contains a unique phosphocompound, di-*myo*-inositol-1,1′-phosphate (DIP), the stereochemistry of which has been deduced (24). DIP was reportedly present in this organism at concentrations equivalent to that of potassium ions (21). DIP has also been found in *Pyrococcus furiosus* (18, 19), in several other species of *Pyrococcus* and *Thermococcus* (19), and in the hyperthermophilic methanogen *Methanococcus igneus* (T_{opt}, 88°C) (8). In *P. furiosus* (18) and *Methanococcus igneus* (8), however, the intracellular DIP concentration was near 50 mM, much lower than that estimated for *P. woesei*. Furthermore, the intracellular concentration of DIP increased for both *Methanococcus igneus* and *P. furiosus* grown at supraoptimal temperatures (8, 18). Notably, DIP (115 mM) increased by 10-fold the stability at 105°C of glyceraldehyde 3-phosphate dehydrogenase purified from *P. woesei* (21). Hence, it was suggested that DIP might have a role as a thermoprotectant (8, 18), although so far DIP has been detected only in hyperthermophilic *Archaea*. Objectives of the present investigation, therefore, included determining whether this compound is present in the hyperthermophilic bacterium *Thermotoya maritima* (T_{opt}, 80°C) (13) and if so, whether its concentration varies with the growth conditions and whether the purified compound affords any thermal protection to enzymes purified from this organism.

T. maritima (DSM 3109) was grown with glucose as the carbon source in a 600-liter fermentor at 80°C with the medium previously described (15), except that $(NH_4)_2CO_3$ (12 mM), KCl (26.8 mM), $CaCl_2$ (0.45 mM), and yeast extract (0.25%, wt/vol) were at the indicated concentrations and NaI, H_3BO_4, $SrCl_2$, and KI were omitted. After the medium was sterilized and cooled to 80°C, dipotassium phosphate (3.7 mM) was added and cysteine hydrochloride (2.3 mM) was used as the reductant. When required, the pH of the culture was maintained at 7.0 (measured at 80°C) by the addition of 5 M NaOH. Cell growth was monitored by measuring the absorbance at 600 nm and by protein determination (6). Growth studies to monitor the effect of ionic strength and growth temperature on the DIP levels in *T. maritima* were performed in the presence of 20 mM thiosulfate (20). DIP was identified and its concentration was determined by one-dimensional proton-decoupled ^{31}P nuclear magnetic resonance (NMR) spectroscopy performed on a Bruker 300 NMR spectrometer. The conditions were as follows: spectrometer frequency, 121.5 MHz; sweep width, 10 kHz; relaxation delay, 3 s; pulse, 45°; receiver gain, 80 to 1,600; transmitter offset frequency, 6,400 Hz; line broadening, 2.5 Hz. Phosphoric acid (85%, vol/vol) was the reference (0 ppm) for chemical shift measurements, and inorganic phosphate (4 to 20 mM) was the internal standard for quantitation. Cell extracts of *T. maritima* were prepared by sonication. These were extracted with ethanol as previously described (16), concentrated by lyophilization, and suspended in D_2O (Sigma, St. Louis, Mo.) for spectroscopic analysis.

Preliminary analyses of ethanol extracts of both fresh and

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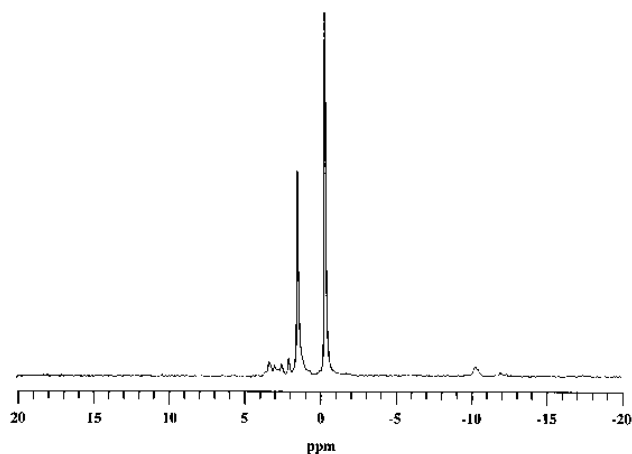

FIG. 1. One-dimensional proton-decoupled ^{31}P NMR spectrum of an ethanol extract of *T. maritima*. The chemical shifts of DIP and P_i are −0.58 and ca. 1.5 ppm, respectively. The minor peaks next to the P_i signal are due to the phosphorylated glycolytic intermediates. Number of scans, 1,024; line broadening, 2.5 Hz.

frozen *T. maritima* cells showed that DIP was indeed present and that DIP and inorganic phosphate were the major intracellular phosphocompounds (Fig. 1). Compounds such as cyclic 2,3-diphosphoglycerate (cDPG), adenosine phosphates, and UDP amino sugars could not be detected. As shown in Fig. 2, under pH-controlled conditions (pH 7.0), the specific amounts of DIP remained fairly constant throughout the growth phase. DIP could not be detected in spent medium,

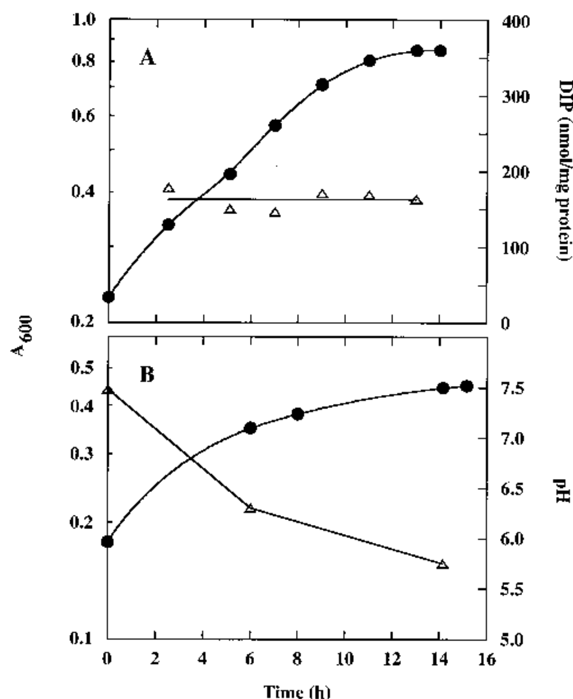

FIG. 2. Growth of *T. maritima* under pH-controlled (A) and non-pH-controlled (B) conditions in a 600-liter fermentor at 80°C. Growth (●) was measured by A_{600}. (A) Approximately 3 liters of medium was removed from the fermentor at the indicated times and rapidly chilled on ice, and the harvested cells were prepared for ^{31}P NMR spectroscopic analysis (△). (B) The extracellular pH (△) of the medium was measured by a pH probe at room temperature.

indicating that it is not secreted. Assuming an intracellular volume of 5 μl/mg of protein, the concentrations of DIP and P_i were estimated to be 32 and 13 mM, respectively. The final cell yield under pH-controlled conditions was approximately 2.4 g (wet weight)/liter. On the other hand, in a non-pH-controlled medium, the cell growth yield was much lower and the pH of the medium decreased to almost 5.5 due to the organic acid end products (22) of fermentation (Fig. 2). The final cell yield was 0.8 g (wet weight)/liter. While the internal phosphate concentration increased to approximately 25 mM, the intracellular DIP concentration remained at 32 mM, showing that its synthesis and degradation are not responsive to the extracellular pH or internal phosphate concentration.

Studies were done to determine the response of DIP levels to the external NaCl concentration and the growth temperature. NaCl-dependent studies were performed at 80°C; temperature-dependent incubation studies (74 to 86.5°C) were performed at an external NaCl concentration of 0.3 M. All experiments were performed in 1-liter Hungate bottles in an argon atmosphere to a volume of ca. 500 ml in controlled water baths and/or in a hot-air convection incubator, and accurate temperature measurements were made by measuring the water temperature in a Hungate bottle maintained in parallel. Cells were grown to a maximal density, rapidly chilled on ice, and centrifuged at 4°C. The cell pellet was suspended in distilled water, and after a freeze-thaw cycle, the cell suspension was sonicated. A portion of this suspension was directly used for ^{31}P NMR spectroscopic analyses and DIP quantitation.

As shown in Fig. 3, the DIP concentration increased as the NaCl concentration in the growth medium increased from 0.2 to 0.4 M and then remained fairly constant up to 0.6 M NaCl. Remarkably, at higher concentrations of NaCl the DIP levels declined sharply. The concentration of DIP in *T. maritima* also increased with growth temperature (Fig. 3). These data show that DIP is accumulated by *T. maritima* in response to both the external salt concentration and the growth temperature, suggesting that this compound may have both a thermoprotectant and an osmolytic role. Similar results with DIP have been reported for the archaea *Methanococcus igneus* (8) and *P. furiosus* (18).

In order to investigate the properties of DIP, a large-scale purification procedure was devised. The cytoplasmic fraction was prepared from 470 g (wet weight) of frozen *T. maritima* cells as previously described (15), except that the buffer was 50 mM Tris HCl (pH 8.0). This was applied to a column (10 by 20 cm) of DEAE Sepharose (Pharmacia LKB, Piscataway, N.J.) equilibrated with the same buffer. DIP was detected only in the material that was not adsorbed. The column was washed with several volumes of the same buffer, and the DIP-containing fraction (8 liters) was concentrated to 500 ml with a rotary evaporator. Since ethanol failed to extract DIP from this sample, a phenol-chloroform (1:1, vol/vol) extraction was performed with the protein pellet, and after centrifugation at 12,000 × *g* at 4°C, the aqueous phase was extracted twice with chloroform to remove phenol; after rotary evaporation, DIP was resuspended in water. To remove contaminating inorganic phosphate, an equimolar concentration of $CaCl_2$ (1.0 M) was added to the concentrated DIP solution, and after incubation for 3 h at 4°C, the precipitate was removed by centrifugation; the procedure was repeated twice. The DIP fraction exhibited a single ^{31}P NMR resonance and was pure as determined by both ^1H and ^{13}C NMR spectroscopy (data not shown); the resonances are identical to those reported for the DIP in *P. woesei* (21) and *Methanococcus igneus* (8). The counterion of DIP prepared in this manner is unknown. The total yield of DIP was 8.4 g. Optical rotation of pure DIP was determined

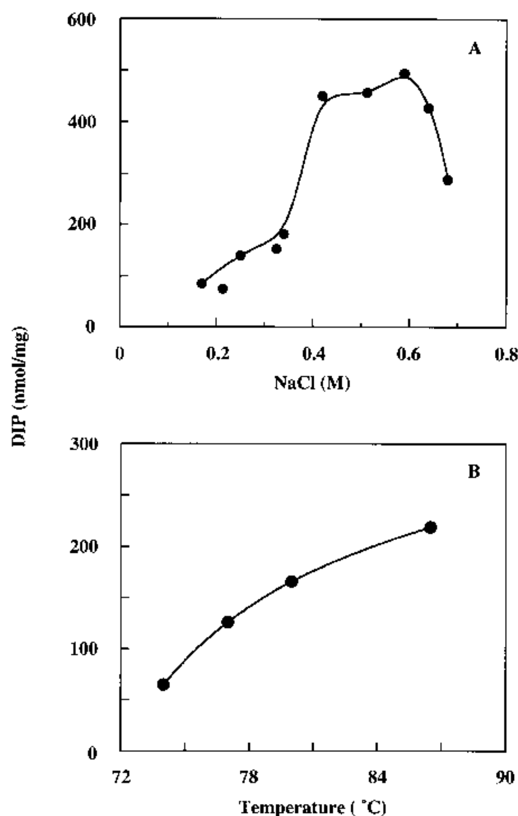

FIG. 3. Response of DIP levels (●) in *T. maritima* to external NaCl concentration (A) and to growth temperature (B). NaCl-dependent studies were performed at 80°C, and temperature-dependent incubation studies (74 to 86.5°C) were performed at an external NaCl concentration of 0.3 M. All experiments were performed as described in the text.

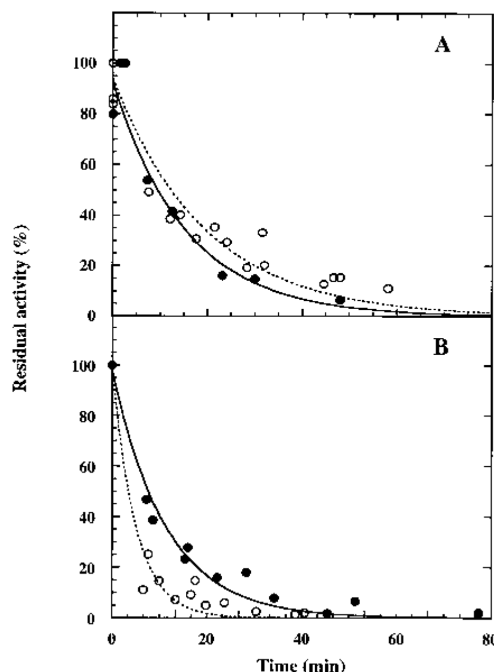

FIG. 4. Stability studies on hydrogenase (A) and POR (B) of *T. maritima* at 90°C. Hydrogenase (0.4 mg/ml) and POR (0.3 mg/ml) in 50 mM EPPS (hydroxyethyl-piperizine propanesulfonic acid) buffer (pH 8.0 at 90°C) containing 2 mM sodium dithionite were incubated in sealed vials under an argon atmosphere at 90°C in the presence of DIP and/or K^+ salt: ●, control (without DIP or K^+); ○, 50 mM DIP with or without 45 mM K^+. Activity was determined at 80°C in a 50 mM EPPS buffer in methyl viologen-coupled spectrophotometric assays. The initial specific activities of hydrogenase and POR were 140 and 61 U · mg^{-1}, respectively. The lines are least-square fits of the data points to a single exponential.

with a polarimeter (Autopol IV; Rudolph Research, Flanders, N.J.). The measurements were made at 20°C in a 250-μl quartz sample holder. The optical rotation of DIP was determined to be $[\alpha]_D^{20} + 0.7°$, suggesting that the DIP in *T. maritima* is an LL isomer similar to the one found in *P. woesei* (24).

The thermal stability of DIP was investigated by incubating the pure material (250 mM, pH 7.3) in D_2O at 90°C within the sample holder of the NMR spectrometer. Proton-decoupled ^{31}P NMR spectra were recorded periodically over a 283-min period. The spectrum of DIP remained unchanged throughout the experiment and no other ^{31}P signals appeared, showing that potential degradative products such as inositol phosphate or inorganic phosphate were not formed. These data indicate that DIP is completely unaffected after approximately 5 h at 90°C.

The efficacy of DIP in minimizing protein denaturation at extreme temperatures was investigated by using two enzymes previously purified from *T. maritima*: pyruvate ferredoxin oxidoreductase (POR) (4) and hydrogenase (15). POR catalyzes the oxidative decarboxylation of pyruvate to acetyl coenzyme A and the electrons are transferred to the redox protein ferredoxin for ultimate disposal as H_2, which is catalyzed by the hydrogenase (1). Both are multimeric, iron-sulfur-containing proteins and both are inactivated by O_2 (4, 15). The pure enzymes were incubated at 90°C under anaerobic conditions with and without DIP (at pH 8.0). As discussed above, potassium ions may have a role in thermal protection in some hyperthermophilic organisms, so these were also included in some experiments by the addition of KCl. Samples were removed periodically from the vials, and the residual enzyme activities were determined spectrophotometrically at 80°C by the pyruvate-dependent (POR) or the H_2-dependent (hydrogenase) reduction of methyl viologen (4, 15). As shown in Fig. 4, the time required for a 50% loss of activity was about 12 min for both enzymes. However, DIP (50 mM), both with and without potassium ions (45 to 180 mM), and potassium ions alone (45 mM) did not significantly stabilize either enzyme, and the results were the same when the DIP concentration was increased to 200 mM. In fact, POR appeared to be less stable in the presence of DIP and/or potassium ions (Fig. 4), and the time required for a 50% loss in activity in the presence of DIP decreased to about 5 min. Similar results were observed with both enzymes when these experiments were performed at pH 7.0 with 50 mM HEPES buffer at 90°C. In this case, the times required for a 50% loss of hydrogenase activity in the absence and presence of DIP were 32 and 37 min, respectively, and for the POR they were 22 and 8 min, respectively.

We have demonstrated that DIP is present in at least one hyperthermophilic bacterium in addition to various hyperthermophilic archaea. Furthermore, its intracellular concentration in *T. maritima* is comparable to those reported for both *P. furiosus* (18) and *Methanococcus igneus* (8) and, as in *P. furiosus* (19), the concentration does not vary throughout the growth phase. The DIP concentration in *T. maritima* is also unaffected by the pH of the growth medium, but it does respond to the growth temperature and external NaCl concentration. Surprisingly, however, the DIP concentration decreased when the NaCl concentration exceeded 0.6 M,

suggesting that DIP is not a universal osmolyte. So far, DIP has been found only in hyperthermophilic organisms, but it is not present in all of them. Hyperthermophilic methanogens such as *Methanothermus fervidus*, *Methanococcus jannaschii*, and *Methanopyrus kandleri* lack DIP and instead contain cDPG as the dominant phosphocompound (11, 17). In addition to having a thermoprotectant role (10), cDPG is involved in gluconeogenesis (9) and presumably in ATP synthesis (23). In contrast to what has been shown with the glyceraldehyde 3-phosphate dehydrogenase of *P. woesei* (21), DIP did not afford any thermal protection to either POR or hydrogenase obtained from *T. maritima*, even though it was present at approximately the physiological concentration in the stability studies. Thus, DIP does not have a general stabilizing effect on purified hyperthermophilic proteins, although the possibility that DIP has an effect on a specific group of proteins cannot be excluded.

However, if it does not play a role in thermal stability, then what is the function of DIP in hyperthermophiles? Unlike cDPG (23), DIP is not thought to be involved in energy metabolism, although some mesophilic aerobes are known to obtain energy from inositol degradation (3). A more likely function is as an osmolyte, because the intracellular concentrations of DIP in *T. maritima* (this work), *Methanococcus igneus* (8), and *P. furiosus* (18) all show a response to the external salt concentration. The data presented herein show that DIP is an extremely thermostable molecule, which would be a prerequisite for such a role. What is surprising, however, is that hyperthermophiles in two distinct phylogenetic domains have utilized this unusual compound. Presumably, they do so for the same reason, but whether this is to protect hyperthermophilic cells from osmotic or thermal stress, or both, remains to be established.

ADDENDUM IN PROOF

Martins et al. (L. O. Martins, L. S. Carreto, M. S. Da Costa, and H. Santos, J. Bacteriol. **178:**5644–5651, 1996) recently identified three different phosphoinositol derivatives in *T. maritima*, one of which is identical to the compound described in this work. During our investigation, we found no evidence for the two additional phosphocompounds (di-*myo*-inositol-1,3′-phosphate and di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate) reported by Martins and coworkers. This may be due to the differences in the extraction procedures that were used in the two studies.

We thank Quincy Teng for assistance with the NMR experiments and Andrea Hutchins for providing *T. maritima* POR. We also acknowledge the assistance of Paul Spearing with the optical rotation measurement. Constructive comments of the anonymous reviewers are gratefully acknowledged.

V.R. is a recipient of a graduate fellowship from the Rotary International Foundation. M.F.J.M.V. was funded in part by a NATO Science Fellowship from the Netherlands Organization for Scientific Research (NWO). The research was supported by grants from the Department of Energy (FG09-88ER13901) and the National Science Foundation (MCB-9405783).

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