Volume 306, number 2,3, 239–242 FEBS 11305 July 1992

Di-myo-inositol-1,1′-phosphate: a new inositol phosphate isolated from *Pyrococcus woesei*

Stefan Scholz[a], Johann Sonnenbichler[b], Wolfram Schäfer[b] and Reinhard Hensel[a]

[a]*FB 9 Mikrobiologie, Universität GHS Essen, Universitätsstr. 5, 4300 Essen 1, Germany* and [b]*Max-Planck-Institut für Biochemie, Am Klopferspitz, 8033 Martinsried, Germany*

Received 21 April 1992; revised version received 1 June 1992

A new inositol derivative could be isolated from the Archaeum *Pyrococcus woesei* and identified as di-myo-inositol-1,1′-phosphate by ^{1}H, ^{31}P NMR spectroscopy, mass spectrometry and thin layer chromatograpy. In *P. woesei*, this inositol phosphate represents the dominant counterion of K^+ which ranges from 500 to 600 mM. The role of the potassium salt of di-myo-inositol-1,1′-phosphate as thermostabilizer is discussed.

Archaea; Thermophile; Protein thermostabilization; Intracellular ion concentration

1. INTRODUCTION

Some hyperthermophilic members of the Archaea (Archaebacteria) exhibit strikingly high intracellular ion concentrations. For instance, in the cells of the hyperthermophilic methanogens *Methanothermus fervidus*, *M. sociabilis* and *Methanopyrus kandleri* high potassium ion concentrations were found ranging from approx. 1 M up to 2.3 M [1,2], counterbalanced by the trianionic cyclic 2,3-diphosphoglycerate (cDPG) [1,3]. The physiological significance of this high intracellular ion concentration is still the subject of speculation. Thus, the role of cDPG as a storage compound has been discussed [4–7]. On the other hand, from the temperature dependence of the intracellular concentration of K^+ and cDPG as well as from the thermostabilizing action of the tripotassium salt of cDPG on proteins [1], it has been suggested that both ions play an important role in thermoadaptation.

Interestingly, unusual high K^+ ions are also present in the cells of *Pyrococcus woesei*, another hyperthermophilic Archaeum growing optimally at 100°C with an upper growth limit at 104°C [8,9]. The corresponding anion, however, remained unidentified. Here we describe the structure of the new organic phosphate which presumably counterbalances the positive charge of K^+ within the *P. woesei* cells.

Correspondence address: R. Hensel, FB 9 Mikrobiologie, Universität GHS Essen, Universitätsstr. 5, 4300 Essen 1, Germany. Fax: (49)(201) 183 2529.

2. MATERIALS AND METHODS

2.1. *Bacteria and growth conditions*

Cells of *P. woesei* were grown at 95°C under nitrogen atmosphere. The growth medium was composed as described by Zillig et al. [8].

2.2. *Determination of intracellular volume and intracellular content of potassium*

The intracellular volume was determined by the centrifugation method [10] using $^{3}H_2O$ and [^{14}C]sucrose for labelling total and extracellular water. Potassium was determined by flame photometry.

2.3. *Determination of phosphate*

Inorganic phophate was determined by the method of Eibl and Lands [11] using the Serva test. The respective samples were pretreated with ethanolic $Mg(NO_3)_2$ solution [12] for quantification of organic phosphate.

2.4. *Preparation of the dominant organic phosphate*

10 g of frozen cells were suspended in 10 ml of distilled water and disrupted by three passages through the French Press cell. After removing the cell debris by centrifugation and deproteination by ethanol precipitation, the extract was applied to a Q-Sepharose (Sigma) column (bed volume: 125 ml). The separation was performed with a linear gradient of 0–0.2 M NH_4HCO_3. The fractions with the highest organic phosphate content (elution at 0.04 M NH_4CO_3) were pooled and lyophilized several times to remove the salt. Further purification was achieved by preparative thin layer chromatography. The plates were coated with a 2 mm-thick layer of silica gel G-60 (Merck). Chromatography was performed with a mixture of chloroform/methanol/25% ammonia (6:10:5). Usually, the plates were developed twice. For analytical purposes organic phosphates were visualized using the Hanes reagent [13]. For preparative purposes, respective areas of the silica gel were scraped off and eluted with water. After lyophilisation the sample was applied to a Sephadex G-10 (Pharmacia) column to remove residual silica material.

The potassium salt of the organic phosphate was prepared by treatment with Dowex 50W.

2.5. *NMR spectroscopy*

The NMR spectra were recorded with a Bruker AM 500 NMR

spectrometer in D_2O. HDO was used as the standard (= 4.80 ppm) for ^1H NMR and 50% H_3PO_4 (= 0 ppm) for ^{31}P NMR.

^1H-resonances (Fig. 2):

H-1 4.07 ppm ddd $J_{1,2}$ = 2.5 Hz $J_{1,6}$ = 9.2 Hz $J_{1,P}$ = 8.0 Hz
H-2 4.33 ppm dd $J_{1,2}$ = 2.5 Hz $J_{2,3}$ = 2.2 Hz
H-3 3.61 ppm dd $J_{2,3}$ = 2.2 Hz $J_{3,4}$ = 9.5 Hz
H-4 3.69 ppm dd $J_{3,4}$ = 9.5 Hz $J_{4,5}$ = 9.5 Hz
H-5 3.38 ppm dd $J_{4,5}$ = 9.5 Hz $J_{5,6}$ = 9.0 Hz
H-6 3.82 ppm dd $J_{5,6}$ = 9.0 Hz $J_{1,6}$ = 9.2 Hz

^{31}P-resonance:

1.08 ppm tr J = 8.0 Hz s with ^1H-BB decoupling

2.6. *Mass spectrometry*

Negative liquid secondary ion mass spectra were obtained with the mass spectrometer MSQ 30 from Finnigan MHT, Bremen, equipped with a cesium gun from HMD Intecta GmbH, Harpstedt. Glycerol was used as matrix, the Cs-ions had 10 kV energy and approx. 4 μA ion current.

2.7. *Alkaline hydrolysis of the organic phosphate*

1 μg of the organic phosphate was hydrolyzed by incubation with approx. 400 μl of 10 N NH_4OH in a sealed glass vessel at 110°C for 18 h. NH_4OH was then removed by evaporation in a Speed Vac concentrator (Savant).

3. RESULTS AND DISCUSSION

3.1. *Identification of the dominant organic phosphate in P. woesei*

Thin layer chromatography of ethanol extracts of *P. woesei* yielded 3 low-molecular weight compounds, giving positive reaction with the Hanes reagent (Fig. 1, lane 1): inorganic phosphate (A), organic phosphate (B) and a not yet identified uncharged organic compound (C), which, however, does not contain phosphate.

The organic phosphate compound was purified to homogeneity by anion exchange chromatography, thin layer chromatography and gel filtration.

From NMR studies, the dominant organic phosphate could be identified as di-myo-inositol-1,1'-phosphate (DIP).

The structure of the polyol has been derived from the shift values and coupling constants in the ^1H NMR spectra (Fig. 2). The connectivities of the resonance lines were confirmed by two dimensional H/H-correlation spectra (COSY). The dieder angles have been calculated using the Karplus equation.

The triplet structure of the ^{31}P resonance (Fig. 3) is the consequence of a dd structure arising from the two adjacent inositol hydrogens with a three-bond spin coupling $^3J_{PH}$ = 8.0 Hz. By broad bond hydrogen decoupling, this signal is coalescing to a singlet.

The mass spectrum showed a M-H ion m/Z 420; this and the fragment m/z 241 ($C_6H_{11}OPO_2$-H) and m/z 259 ($C_6H_{11}OPO_3H$) are in agreement with the proposed structure (Fig. 3).

Further evidence for the correct structure came from hydrolysis experiments. As expected from the proposed structure, alkaline hydrolysis yields inositol and inositol phosphate as analyzed by thin layer chromatography (Fig. 1, lane 5). The stereochemical configuration of

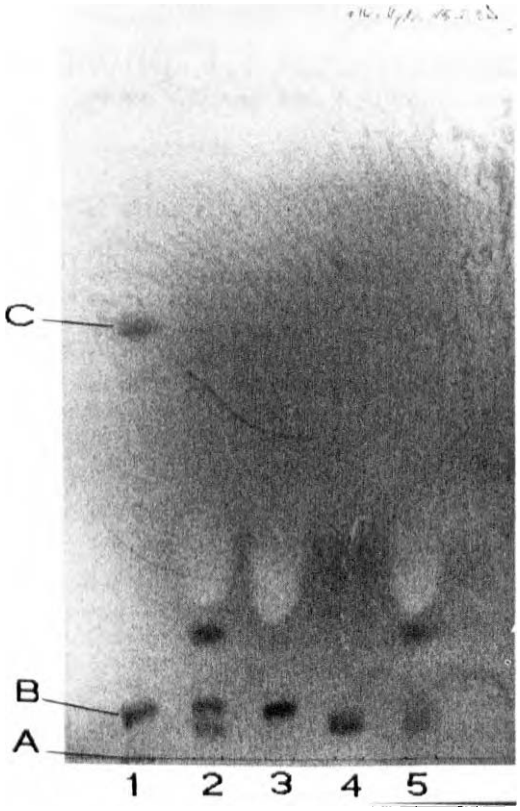

Fig. 1. Thin layer chromatography of ethanol extract of *P. woesei*. (Lane 1) Ethanol extract of *P. woesei*; (lane 2) co-chromatography of purified DIP, myo-inositol (top) and myo-inositol-1-phosphate; (lane 3) purified DIP; (lane 4) myo-inositol-1-phosphate; (lane 5) alkaline hydrolysis of DIP.

myo-inositol-1-phosphate, however, remains to be determined.

DIP represents a new inositol derivative. Another inositol derivative from Archaea, L-myo-inositol-1-phosphate, has already been described as the polar headgroup moiety of membrane ether lipids [14,15]. Thus, like Eucarya but unlike Bacteria, members of Arechaea are able to synthesize inositol.

Studies on the biosynthesis of inositol and its derivative DIP in *P. woesei* are currently underway.

3.2. *Intracellular concentration of K^+ and DIP in P. woesei*

Due to the high susceptibility of *P. woesei* to cell lysis, the intracellular K^+ concentration could only be determined rather inaccuratly. Concentration values ranging from 500 to 600 mM were obtained from five indepentent measurements.

Attempts to quantify the intracellular DIP concentration were performed in crude extracts using ^{31}P NMR spectroscopy with 2 mM glucose-6-phosphate as the internal standard. The dilution factor was determined for calculating the intracellular concentration from the K^+ content of the crude extract. The deduced DIP con-

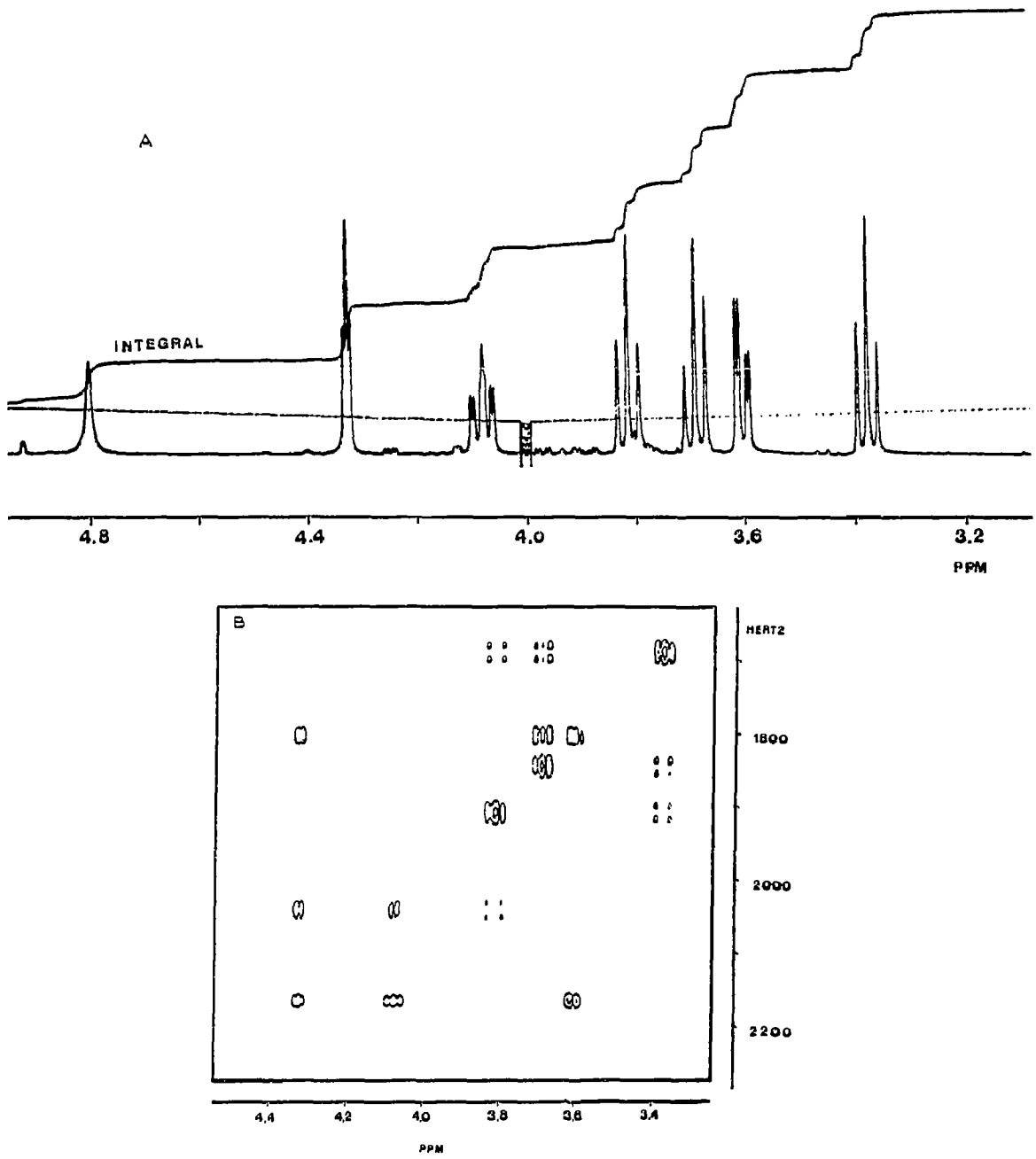

Fig. 2. (A) ^{1}H NMR spectrum of the dominant organic phosphate in *P. woesei*. (B) H/H-correlation spectrum (cosy) of di-myo-inositol-1,1'-phosphate in D_2O.

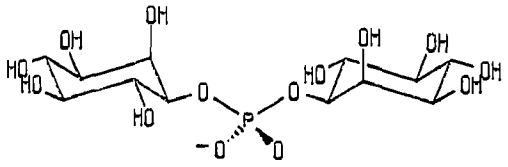

Fig. 3. Structure model of L-di-myo-inositol-1–1'-phosphate, one of the two stereoisomers of di-myo-inositol-1–1'-phosphate.

centration ranges from 500 to 900 mM. Therefore, we speculate that DIP represents the main counter ion of K^+ in *P. woesei* cells. To confirm this assumption, however, a more detailed analysis of the intracellular cation and anion content of *P. woesei* must be performed.

3.3. *Thermostabilizing action of DIP*

As shown in a previous publication [9], the intrinsic

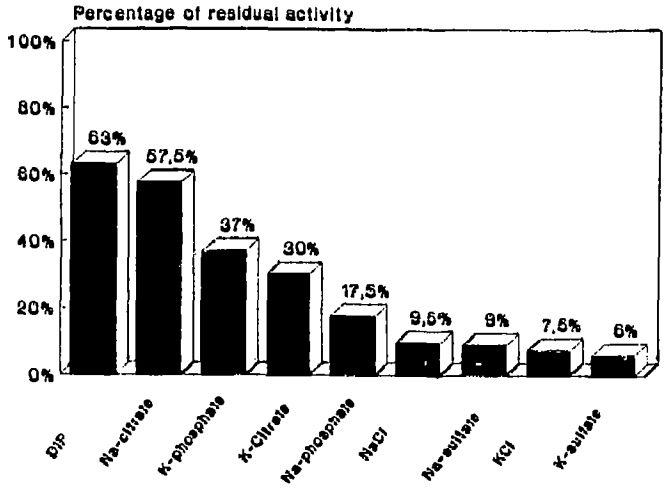

Fig. 4. Thermostabilization of the *P. woesei* GAPDH by DIP: comparison of the stabilizing action to other potassium and sodium salts. The incubation was performed for 30 min at 105°C under anaerobic conditions. The enzyme concentration was 80 μg/ml, the concentration of the salts 115 mM.

thermostability of the glyceraldehyde-3-phosphate dehydrogenase of *P. woesei* is too low for the growth range up to 104°C. In order to test whether K^+ and its presumed counterion DIP are able to stabilize the enzyme against thermoinactivation, the enzyme was incubated in the presence of the potassium salt of DIP. The test conditions were the same as described by Zwickl et al. [9]. Limited by the available amount of DIP, the stability tests could only be performed with salt concentrations up to 115 mM. As compared to other salts, the potassium salt of DIP proved to be the most efficient, also at a concentration sigificantly lower than that in vivo (Fig. 4). Thus, we assume that the potassium salt of DIP acts as thermostabilizer in vivo. However, we cannot exclude that this new inositol derivative also performs other functions, e.g. as phosphate or carbon reservoir.

Acknowledgements: We thank Mr. S. Wunderl at the Max-Planck-Institut für Biochemie for growing the *P. woesei* cultures. We are also indebted to Dr. I. Zetl for assistance in performing the NMR measurements. This work was supported by grants of the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

REFERENCES

[1] Hensel, R. and König, H. (1988) FEMS Microbiol. Lett. 49, 75–79.
[2] Lehmacher, A. and Hensel, R. (1990) DECHEMA Biotechnology Conferences 4, 415–418; VCH Verlagsgesellschaft
[3] Huber,R., Kurr, M., Jannasch, H.W. and Stetter, K.O. Nature 342, 833–834.
[4] Kanodia, S. and Roberts, M.F. (1983) Proc. Natl. Acad. Sci. USA 80, 5217–5221.
[5] Seely, R.J. and Fahrney, D.E. (1983) J. Biol. Chem. 258, 10835–10838.
[6] Gorris, L.G.M., Korteland, J., Derksen, R.J.A.M., Van der Drift, C. and Vogels, G.D. (1990) J. Chromatogr. 504, 421–428.
[7] Evans, J.N.S., Raleigh, D.P., Tolman, C.T. and Roberts, M.F. (1986) J. Biol. Chem. 261, 5693–5698.
[8] Zillig, W., Holz, I., Klenk, H.-P., Trent, J., Wunderl, S., Janekovic, E., Imsel, E. and Haas, B. (1989) Syst. Appl. Microbiol. 9, 62–70.
[9] Zwickl,P., Fabry, S., Bogedain, Ch., Haas, A. and Hensel, R. (1990) J. Bacteriol. 172, 4329–4338.
[10] Stock, J.B., Ranch, B. and Roseman, S. (1977) J. Biol. Chem. 252, 7850–7861.
[11] Eibl, H. and Lands, W.E.A. (1969) Anal. Biochem. 30, 51–57.
[12] Ames, B.N.(1966) Methods Enzymol. 8, 115–118.
[13] Hanes, C.S. and Isherwood, F.A. (1952) Nature 164, 1107.
[14] Lanzotti, Trincone, A., Nicolaus, B., Zillig, W., De Rosa,M. and Gambacorta, A. (1989) Biochim. Biophys. Acta 1004, 44–48.
[15] Langworthy, T.A., Smith, P.F. and Mayberry, R.W. (1974) J. Bacteriol. 119, 106.