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Quantification of cyclic 2,3-diphosphoglycerate from methanogenic bacteria by isotachophoresis

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SUMMARY

Cyclic 2,3-diphosphoglycerate (cDPG), a metabolite exclusively present in methanogenic bacteria, was separated from other phosphate-containing compounds in extracts of *Methanobacterium thermoautotrophicum* by means of isotachophoresis. The detection limit was 100 pmol per injected sample. Quantification of intracellular concentrations of cDPG with the isotachophoretic assay and with a spectrophotometric cDPG assay gave comparable results, but the isotachophoretic assay was faster, less laborious and more sensitive. A possible involvement of cDPG in the energy metabolism was indicated by studies of *Mb. thermoautotrophicum* in batch and continuous cultures.

INTRODUCTION

In 1983 a unique cyclic pyrophosphate was isolated from *Methanobacterium thermoautotrophicum*, the structure of which was identified[1–3] as the intramolecularly cyclized pyrophosphate of 2,3-diphospho-D-glycerate (Fig. 1), referred to in the following as cDPG. Under optimum growth conditions, this unusual metabolite is the major soluble carbon- and phosphorous-containing compound in cells of *Mb. ther-*

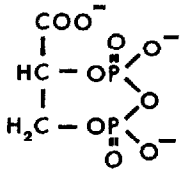

Fig. 1. Structure of cyclic 2,3-diphospho-D-glycerate (cDPG).

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moautotrophicum. Intracellular concentrations have been found to range from 2 to 200 m*M* under different growth conditions[3,6].

Up to now, cDPG has been found in significant concentrations in methanogenic bacteria belonging to the genera *Methanobacterium, Methanobrevibacter, Methanothermus* and *Methanosphaera*, whereas it has not been detected in members of the genera *Methanococcus, Methanosarcina* and *Methanogenium*[4,5,7]. One important difference between these two groups of methanogens is that the former possess a rigid pseudomurein cell wall, whereas the latter, with the exception of *Methanococcus*, do not. This might indicate a possible involvement of cDPG in the carbohydrate metabolism as an intermediate in the synthesis of the pseudomurein component of the cell wall. However, several studies point to a multi-functional role of cDPG in the metabolism of methanogens. For instance, cDPG might be involved in energy storage as it contains a high-energy phosphoryl group which could be utilized in ATP synthesis[1]. In addition, it has been suggested to function as a phosphate storage compound, comparable to polyphosphates in other bacteria, as the intracellular cDPG pool was found to be depleted during growth in the absence of exogenous phosphate[2,6]. cDPG has also been linked to phospholipid synthesis[6] and gluconeogenesis[8]. Finally, the potassium salt of the trianionic cDPG has been found to stabilize the thermolabile enzymes glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase *in vitro*[4].

The spectrophotometric cDPG assay most commonly used, which employs acid hydrolysis of cDPG to 2,3-diphosphoglycerate (2,3-DPG) and subsequent quantification of 2,3-DPG using a multi-enzyme assay[3], is laborious and time consuming. As isotachophoresis has been found to be well suited for accurate quantification of ionic metabolites from methanogens[9], we adopted this fast and simple technique as the basis for a novel cDPG assay. The assay was developed using authentic cDPG obtained by isolation from mass-cultured cells. For reference, cDPG was prepared from 2,3-DPG by organic synthesis. The assay was employed to study the possible role of cDPG in the energy and phosphate metabolism of *Mb. thermoautotrophicum* by investigating the relationship between growth and intracellular levels of cDPG under different physiological conditions.

EXPERIMENTAL

Organism and growth conditions

Mb. thermoautotrophicum strain △H (DSM 1053) was cultured at 65°C under hydrogen–carbon dioxide (80:20, v/v) in a medium composed of (per litre) KH_2PO_4, 6.8 g; $Na_2CO_3 \cdot 10H_2O$, 9.0 g; NH_4Cl, 2.1 g; L-cysteine · HCl, 0.6 g; $Na_2S \cdot 9H_2O$, 0.6 g; and minerals solution, 2 ml. The latter contained (per litre) nitrilotriacetic acid, 96 g; $MgCl_2 \cdot 6H_2O$, 41 g; $MnSO_4 \cdot H_2O$, 5 g; $FeCl_2 \cdot 4H_2O$, 5 g; $NiCl_2 \cdot 6H_2O$, 1.2 g; $ZnSO_4 \cdot 7H_2O$, 1 g; $CoCl_2 \cdot 6H_2O$, 1 g; $CaCl_2 \cdot 2H_2O$, 1 g; H_2SeO_3, 0.8 g; $NaMoO_4 \cdot 2H_2O$, 0.24 g; $CuSO_4 \cdot 5H_2O$, 0.1 g; $AlK(SO_4)_2 \cdot 2H_2O$, 0.1 g; H_3BO_3, 0.1 g; and $NaWO_4 \cdot 2H_2O$, 3 mg. The pH of the culture medium and of the minerals solution was adjusted to 7.0. Cells were grown in a 0.5-l fermentor operated as a batch or continuous culture with hydrogen–carbon dioxide at a flow-rate of 22.4 l/h. Cell growth was monitored by reading the absorbance at 578 nm with a Hitachi Model 191 spectrophotometer and by measuring methane production by gas chromatogra-

phy[10]. Cells were harvested, suspended (10:1, w/v) in 10 m*M* N-tris(hydroxymethyl) methyl 2-aminoethanesulphonate (TES) buffer at pH 7.0 and, following centrifugation, resuspended in this buffer to a minimum concentration of 4 mg dry weight (dw)/ml. Cell-free extracts were prepared from these suspensions as described previously[9]. Crude cell extracts were obtained by boiling (45 min, 100°C) or sonication (seven times 10 s at 70 W; Branson Sonifier B-12) of 0.5- and 1.5-ml samples of cell suspensions, respectively. The intracellular volume of the cells was assumed to be 1.8 ml/g dw of cells[11].

Isotachophoresis

Isotachophoretic separations were performed at ambient temperature with an LKB 2127 Tachophor carrying a 28-cm Teflon capillary tube of 0.4 mm I.D. The leading electrolyte solution contained chloride (10 m*M*) as the leading anion and 6-amino-*n*-hexanoic acid as the buffering counter ion at pH 4.5. Poly(vinyl alcohol) (0.05%) was included in the leading electrolyte to sharpen the zone boundaries. The terminal electrolyte was acetate (10 m*M*) at pH 4.8. The driving current was 500 μA at 3–4 kV. The total assay time was about 15 min. Detection was effected with an a.c. conductivity detector[12]. Zone lengths of eluting anions were derived from the differential signal of the conductivity meter and expressed in units of time (s). Reference solutions were prepared in Milli-Q deionized water (Millipore). Aliquots of 1–5 μl were subjected to isotachophoresis. Calibration graphs were constructed by plotting the zone lengths against the amounts of anion injected.

Spectrophotometric cDPG assay

The assay was performed essentially as described previously[3] with 200–μl samples of cell extracts, containing 0.5–1.0 mg dw of cells. Briefly, the samples were treated with 1 *M* hydrochloric acid (1:1, v/v) for 15 min at 100°C in order to hydrolyse the pyrophosphate bond in cDPG, resulting in a complete conversion to 2,3-DPG[2]. 2,3-DPG was quantified by use of the Boehringer (Mannheim, F.R.G.) commercial assay, which measures the amount of NADH oxidized during the conversion of 2,3-DPG to glycerol-3-phosphate catalysed by five consecutive enzymatic reactions.

Isolation and purification of cDPG from Mb. thermoautotrophicum

Cells were mass-cultured in a 10-l fermentor under hydrogen–carbon dioxide (80:20, v/v; 100 l/h) and harvested at the end of the logarithmic phase of growth. All of the subsequent manipulations were performed at 4°C. Cells were suspended (1:1, w/v) in buffer consisting of 100 m*M* TES and 10 m*M* EDTA at pH 7.2. The cells were broken by passage through a French press at 138 MPa. Perchloric acid (2.1%, w/v) was added to the suspension. After vigorous shaking for 20 min, the suspension was centrifuged at 12 000 *g* for 10 min. The supernatant obtained was neutralized, whereupon centrifugation was repeated. cDPG was purified from the supernatant fraction using a DEAE-Sephadex A-25 column (20 cm × 3 cm I.D.), eluted with a linear gradient of 0.3–1.5 *M* ammonium acetate at pH 7.5, followed by a QAE-Sephadex A-25 column (45 cm × 1.5 cm I.D.), eluted with a linear gradient of ammonium acetate at pH 5. cDPG was traced and quantified in the eluent fractions by spectrophotometric assay. The fractions containing cDPG were pooled, lyophilized and taken up in deionized water. Judged by isotachophoretic analysis, the product obtained was pure.

Chemical synthesis of cDPG

cDPG was prepared by incubating 500 μmol of 2,3-DPG, dissolved in 10 ml of 0.5 *M* Bis–Tris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane] buffer containing 0.2 *M* $MgCl_2$ (pH 6.5), in the presence of 1 *M* EDAC [1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride]. cDPG formation was monitored by use of the isotachophoretic assay. After incubation for 2 days at 40°C with constant shaking, 2,3-DPG conversion to cDPG ceased. The reaction mixture was adjusted to pH 7.5 with ammonia and diluted 100-fold with deionized water. cDPG was isolated from this solution by use of the ion-exchange chromatographic procedure outlined above.

Chemicals

EDAC, Bis–Tris and TES were obtained from Sigma (St. Louis, MO, U.S.A.), poly(vinyl alcohol) from Merck (Darmstadt, F.R.G.), 2,3-diphosphoglycerate from Boehringer and 6-amino-*n*-hexanoic acid from Serva (Heidelberg, F.R.G.).

RESULTS

Isolation of authentic cDPG

A mass culture of *Mb. thermoautotrophicum*, about 3.8 g dw of cells, was used for the isolation of cDPG. Judged by the spectrophotometric assay, the intracellular cDPG content was 160 μmol/g dw of cells (89 m*M*), which is comparable to values reported by others[3,5,6]. Isolation of cDPG from perchloric acid-treated cells by ion-exchange chromatography yielded 120 μmol/g dw of cells extracted. Hence 75% of the cDPG contained in the whole cells was recovered.

Chemical synthesis of cDPG

Incubation of 500 μmol of 2,3-DPG with EDAC–$MgCl_2$, which catalyzes the intramolecular cyclization process, resulted in the formation of 50 μmol of cDPG. Although this is a low transformation yield, the conversion proceeds rapidly (48 h), and can be performed with large amounts of 2,3-DPG to obtain any desired amount of pure cDPG.

Isotachophoretic assay of cDPG and related compounds

The separation obtained with the isotachophoretic assay of a mixture of pure compounds, *i.e.*, cDPG and five other phosphate-containing metabolites, is illustrated in Fig. 2A. It can be seen that each of the compounds has a characteristic position in the isotachopherogram, referred to as the relative step height. In addition to the compounds shown, the assay is suitable for the separation of tripolyphosphate, phosphoenolpyruvate and pyruvate, which have relative step heights of 0.16, 0.29 and 0.40, respectively. Fig. 2B shows a typical isotachopherogram of a cell-free extract of *Mb. thermoautotrophicum* cells. cDPG, P_i and 2,3-DPG were present at millimolar concentrations in the cells, whereas other phosphate-containing compounds possibly present were below the limit of detection at the recorder setting employed. Quantification of cDPG in crude cell extracts prepared by boiling or sonication yielded identical results.

Calibration graphs were prepared for each of the phosphate-containing metab-

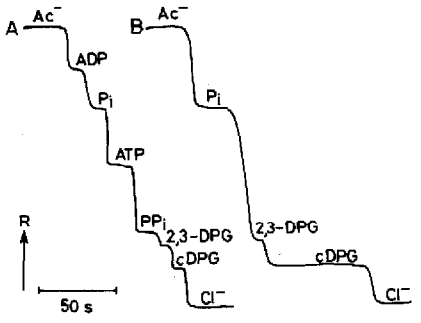

Fig. 2. Isotachopherograms of (A) a reference mixture of phosphate-containing metabolites (1 μl sample volume; cDPG and 2,3-DPG at 1 m*M*, the other ions at 2 m*M*) and (B) a cell-free extract prepared from cells of *Mb. thermoautotrophicum* harvested during logarithmic-phase growth in batch culture (1 μl, containing about 30 μg dw of cell material). Chloride was used as the leading ion and acetate as the terminating ion. 1 = Chloride (0.0); 2 = cDPG (0.13); 3 = 2,3-DPG (0.23); 4 = PP_i (inorganic pyrophosphate) (0.26); 5 = ATP (0.50); 6 = P_i (inorganic orthophosphate) (0.70); 7 = ADP (0.85); 8 = acetate (1.0). Values in parentheses are relative step heigths. R = Resistance.

olites under investigation. A linear relationship was found in all instances between the zone lengths and the amounts of compound injected. In Fig. 3 this is shown for P_i, 2,3-DPG and cDPG. Linearity was good up to about 20 nmol per injection. In all instances the zone lengths were found to be proportional to the amounts of compound injected. Zone lengths recorded for equimolar amounts of the metabolites varied in the proportions P_i:2,3-DPG:cDPG = 1:1.5:2. The limit of detection was in the range 50–200 pmol per injection for the various metabolites, whereas the detecton

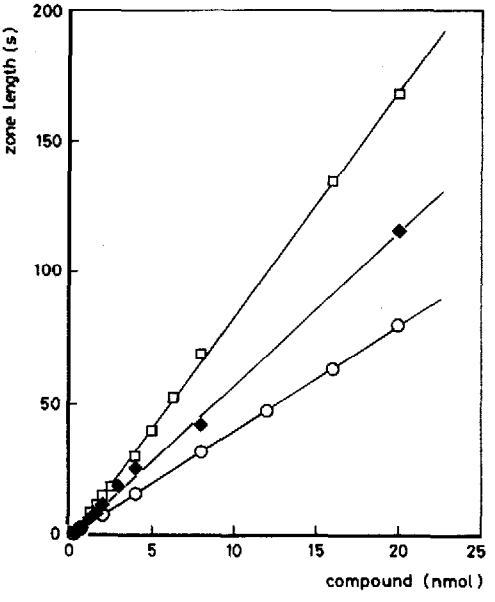

Fig. 3. Calibration graphs indicating the dependence of the zone lengths on the amounts of (○) P_i, (◆) 2,3-DPG and (□) cDPG injected. Solid lines are linear regression curves. All linear regression coefficients were $\geqslant$0.996.

TABLE I

CELLULAR LEVELS OF cDPG AND 2,3-DPG AT DIFFERENT SPECIFIC GROWTH RATES, ASSESSED BY SPECTROPHOTOMETRIC AND ISOTACHOPHORETIC ASSAYS

Cells of *Mb. thermoautotrophicum* were grown in a hydrogen-limited continuous culture with cysteine (0.35 g/l) and thiosulphate (1 g/l) as the source of sulphur.

Specific growth rate (h^{-1})	*Intracellular content (μmol/g dw)*			
	Spectrophotometric assay		*Isotachophoretic assay*	
	cDPG	*2,3-DPG*	*cDPG*	*2,3-DPG*
0.036	210	23	204	12
0.108	111	31	173	30
0.180	113	37	105	24
0.252	79	12	90	7
0.288	70	3	84	4

limit of the spectrophotometric assay is 2 μmol/g dw of cells[6], which is equivalent to about 20 nmol per assayed sample. Results obtained with the isotachophoretic assay were generally in good agreement with those of the spectrophotometric assay, as is shown in Table I for levels of cDPG and 2,3-DPG measured in samples of *Mb. thermoautotrophicum* cultured at different specific growth rates in a continuous culture.

Growth and cDPG levels

Mb. thermoautotrophicum was cultivated batchwise to assess growth and intracellular levels of cDPG as a function of time (Fig. 4). Growth proceeded exponentially up to cell densities of absorbance 0.8. At absorbance values of 0.8–2.0 growth was linear, probably as a result of hydrogen limitation due to inefficient hydrogen transfer into the culture medium[6]. From Fig. 4 it can be seen that high levels of cDPG

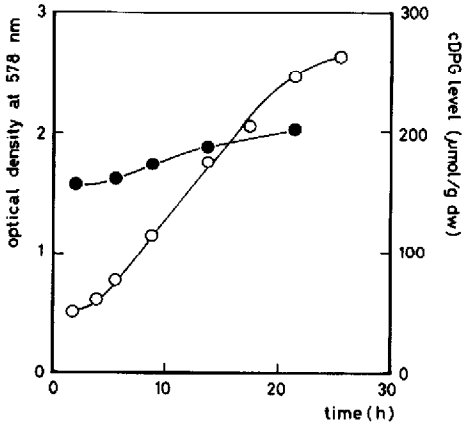

Fig. 4. Relationship between growth and cellular cDPG content of cells of *Mb. thermoautotrophicum* grown in a 0.5-l batch culture. ○ = Absorbance at 578 nm; ● = cDPG concentration. dw = Dry weight.

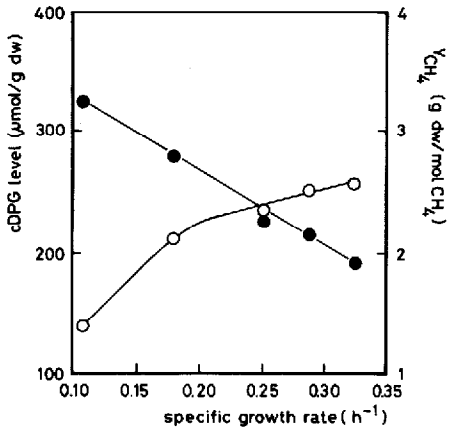

Fig. 5. (●) Intracellular cDPG level and (○) specific growth yield (Y_{CH_4}) in relation to the specific growth rate in a hydrogen-limited chemostat culture of *Mb. thermoautotrophicum*.

are maintained in stationary- and logarithmic-phase cells and also in cells during linear growth. A relatively small increase in the cDPG concentration was recorded during the course of growth.

To determine the cellular level of cDPG in relation to the yield of growth at different specific growth rates, *Mb. thermoautotrophicum* was cultured in a hydrogen-limited chemostat under steady-state conditions at different dilution rates. The values obtained for both parameters are given in Fig. 5. The concentration of cDPG was found to increase with decreasing growth rate, an observation substantiated by the data in Table I, while the yield of cells per mole of methane produced (Y_{CH_4}) decreased with decreasing growth rate.

DISCUSSION

Isotachophoresis can be used for the qualitative and quantitative analysis of anionic metabolites[9,12]. Sample components are introduced at the interface of a discontinuous buffer system, the leading and terminating electrolytes, and separated into discrete, consecutive zones with homogeneous concentrations. Each compound can be identified by its relative step height in the isotachopherogram, and its concentration can be derived from the associated zone length. Isotachophoresis is a simple and rapid technique and was found here to be very well suited to measure the concentration of phosphate-containing metabolites in cell extracts of a methanogenic bacterium. Quantification of cDPG and 2,3-DPG with the novel isotachophoretic assay yielded values that were in good agreement with those obtained with the spectrophotometric assay commonly employed for this purpose. With the isotachophoretic assay, however, the detection limit is two orders of a magnitude lower and the manipulations are less elaborate and less time consuming in comparison with the spectrophotometric assay.

cDPG is one of several unusual metabolites unique to methanogenic bacteria[13]. As cDPG levels as high as 200 μmol/g dw, equivalent to 5% of the total dry weight of the cells, have been found in *Mb. thermoautotrophicum*, an important physiological

function of cDPG is evident. Here we investigated possible functions of cDPG in the phosphate and energy metabolism of this methanogen, using the isotachophoretic assay for quantification.

During batch cultivation, high concentrations of cDPG were maintained both in logarithmic-phase cells and in stationary-phase cells. Apparently, intracellular levels of cDPG are not strongly influenced by the metabolic state of the cell. Although cDPG has been suggested[2,6] to function as a phosphate storage compound, the observation that cDPG levels do not increase significantly during stationary growth, as polyphosphate levels in other microorganisms do, does not support this view.

In hydrogen-limited chemostat cultures, an increase in the cellular content of cDPG was observed with a decrease in specific growth rate. The latter phenomenon might be related to changes in the energy metabolism of the cell and could indicate a function of cDPG as an energy-storage compound in *Mb. thermoautotrophicum*. Assuming that the amount of ATP synthesis is comparable at different growth rates, the higher availability of ATP at a low growth rate could then account for a lower cDPG turnover, and hence the higher cDPG concentrations found in the cells at low rates of growth. A role of cDPG as phosphagen, however, does not find much support in the literature[1], and several groups have reported results to the contrary of such a function[2,5,6,8].

The question of why *Mb. thermoautotrophicum* and a number of other methanogenic bacteria maintain high intracellular levels of cDPG remains unanswered. The relevance of the various functions attributed to this compound needs to be substantiated by future research, which might be facilitated by the isotachophoretic cDPG assay presented here.

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