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Biosynthesis of cyclic 2,3-diphosphoglycerate

Isolation and characterization of 2-phosphoglycerate kinase and cyclic 2,3-diphosphoglycerate synthetase from *Methanothermus fervidus*

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Starting from 2-phosphoglycerate the biosynthesis of cDPG comprises two steps: (i) the phosphorylation of 2-phosphoglycerate to 2,3-diphosphoglycerate and (ii) the intramolecular cyclization to cyclic 2,3-diphosphoglycerate. The involved enzymes, 2-phosphoglycerate kinase and cyclic 2,3-diphosphoglycerate synthetase, were purified form *Methanothermus fervidus*. Their molecular and catalytic properties were characterized.

cyclic 2,3-diphosphoglycerate; 2-Phosphoglycerate kinase; cyclic 2,3-diphosphoglycerate synthetase; Methanogen

1. INTRODUCTION

Cyclic 2,3-diphosphoglycerate (cDPG) occurs in several methanogenic genera of the archaebacteria in a wide concentration range from 1 mM up to 1 M [1–7]. Its unusual structure is characterized by an intramolecular high-energy pyrophosphate linkage [1,2,8]. The structural features and the analysis of the cDPG-pool under different growth conditions of *Mb. thermoautotrophicum* [4,9,10] has led to conflicting suggestions of the function of cDPG as a storage compound for energy [1,4], carbon [11] or phosphorus [2].

The highest intracellular concentrations of cDPG were detected in the hyperthermophilic genera *Methanothermus* and *Methanopyrus* [5,6] and for *Mt. fervidus* the concentration increases up to the optimal growth temperature [5]. From this we conclude that cDPG is involved in the thermoadaptation. This suggestion is supported by in vitro stabilization of enzymes from *Mt. fervidus* [5].

The biosynthesis and degradation of cDPG has not yet been analyzed. It has been suggested that cDPG is formed from phospho*enol*pyruvate by hydrolyzing ATP and subsequently decays to 2,3-diphosphoglycerate (2,3-DPG) [11,12]. From labelling experiments with crude extract from *Mb. thermoautotrophicum* it was presumed that cDPG takes part in the carbohydrate metabolism and represents an obligatory gluconeogenetic intermediate.

For the better understanding of the physiological function of cDPG we investigated its biosynthesis in *Mt. fervidus* and characterized the enzymes involved.

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Abbreviations: cDPG, cyclic 2,3-diphosphoglycerate; cDPGS, cyclic 2,3-diphosphoglycerate synthetase; 2,3-DPG, 2,3-diphosphoglycerate; 2-PG, 2-phosphoglycerate; 2PGK, 2-phosphoglycerate kinase

2. MATERIALS AND METHODS

2.1. *Bacteria and chemicals*

Cells of *Mt. fervidus* DSM 2088 were a gift from K.O. Stetter (Universität Regensburg). 2,3-DPG-agarose was synthesized using the coupling protocol of Pharmacia for AH-Sepharose 4B. Q-Sepharose, ATP-agarose, 2-phosphoglycerate, 2,3-diphosphoglycerate, acid phosphatase and enolase were purchased from Sigma; the test kit for 2,3-DPG, ATP and pyruvate kinase was bought from Boehringer Mannheim; ADP-agarose was from Pharmacia; Chelex 100 from Bio-Rad; cellulose thin layer plates were purchased from Merck; hydroxyapatite was obtained from Fluka and [γ-^{32}P]ATP from Amersham. All other chemicals were of analytical grade from Sigma.

2.2. *Enzyme assays*

2.2.1. 2-Phosphoglycerate kinase (2PGK) from *Mt. fervidus*

The buffer used in this and the following assay of cyclic 2,3-diphosphoglycerate synthetase (cDPGS) was 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.5. The activity of 2PGK was determined by measuring the amount of NADH oxidized during the conversion of the 2,3-DPG (formed from 2-PG) to glycerol-3-phosphate. 40 μl of enzyme solution were incubated with 10 μl of a substrate mixture containing 50 mM $MgCl_2$, 50 mM ATP and 25 mM 2-PG at 75°C for 10 min. 10 μl of the reaction mixture were tested for 2,3-DPG using the Boehringer assay kit. One unit (U) of 2PGK produces one μmol 2,3-DPG per min at 75°C.

The reverse reaction was tested with 50 mM 2,3-DPG, 10 mM $MgCl_2$, and 10 mM ADP using the procedure described above.

2.2.2. Cyclic 2,3-diphosphoglycerate synthetase (cDPGS) from *Mt. fervidus*

The activity of cDPGS was determined from the amount of cDPG formed from 2,3-DPG. 40 μl of the enzyme solution were incubated

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with 10 μl of a substrate mixture containing 50 mM $MgCl_2$, 50 mM ATP, 250 mM 2,3-DPG and 2.5 M KCl at 75°C for 10 min. The non-converted 2,3-DPG was hydrolyzed with 10 U acid phosphatase with the addition of 10 μl of 500 mM sodium acetate buffer, pH 4.0, at 37°C for 1 h. The completion of the hydrolysis was controlled with the Boehringer assay kit for 2,3-DPG. The samples free of 2,3-DPG were incubated with 200 μl of 1 M HCl in boiling water to hydrolyze the produced cDPG to 2,3-DPG. After 15 min the samples were neutralized with 200 μl of 1 M Na_2CO_3 and the 2,3-DPG concentrations of aliquots were determined with the Boehringer kit. One unit (U) of cDPGS forms one μmol cDPG per min at 75°C.

The reverse reaction was measured in 300 mM cDPG, 10 mM $MgCl_2$, 10 mM ADP, 500 mM KCl and 20 mM potassium phosphate buffer, pH 7.0, as described above.

2.3. *Enzyme purifications*

2.3.1. 2-Phosphoglycerate kinase from *Mt. fervidus*

All buffers used in the purification procedures of 2PGK and cDPGS contained 2 mM DTE and 200 μM PMSF. 10 g (wet) cells of *Mt. fervidus* (DSM 2088) were suspended in 30 ml of 100 mM potassium phosphate buffer, pH 6.5 and sonicated for 20 min in intervals of 1 min. Cell debris was separated by centrifugation at 37000 × *g* for 30 min and the supernatant was applied to a hydroxyapatite column (2.5 × 4 cm; equilibrated with a 100 mM potassium phosphate buffer, pH 6.5). The column was washed with 450 mM potassium phosphate buffer, pH 6.5 and the 2PGK was subsequently eluted with 850 mM potassium phosphate buffer, pH 6.5. The eluate was dialyzed overnight against 100 mM potassium phosphate buffer, pH 7.5 and then applied to an ADP-agarose column (1 × 5 cm; equilibrated with 100 mM potassium phosphate buffer, pH 7.5). This column was washed first with 100 mM potassium phosphate buffer, pH 7.5, containing 3 M KCl and subsequently with 50 mM potassium phosphate buffer, pH 7.5, containing 7.5 mM ATP. Finally the pure 2PGK was eluted with 100 mM potassium phosphate buffer containing 7.5 mM ATP.

2.3.2. Cyclic 2,3-diphosphoglycerate synthetase from *Mt. fervidus*

The crude extract prepared by the same procedure as described for 2PGK using 50 mM potassium phosphate buffer, pH 6.5, was applied to a Q-Sepharose column (2.5 × 8 cm; equilibrated with 50 mM potassium phosphate buffer, pH 6.5). After washing the column with 50 mM potassium phosphate buffer, pH 6.5, containing 100 mM KCl, the cDPGS was eluted with the same buffer containing 250 mM KCl. The eluate was loaded on a hydroxyapatite column (2.5 × 4 cm; equilibrated with 150 mM potassium phosphate buffer, pH 6.5). After rinsing the column with the equilibration buffer, cDPGS was eluted with 350 mM potassium phosphate buffer, pH 6.5 and dialyzed against 10 mM potassium phosphate buffer, pH 7.0. The dialyzed eluate was applied to an ATP-agarose column (2 × 9 cm; equilibrated with 50 mM potassium phosphate buffer, pH 7.0) subsequently rinsed with the equilibration buffer. cDPGS was eluted with the same buffer containing 2 mM ATP and then loaded on a 2,3-DPG-agarose column (1 × 13 cm; equilibrated with 50 mM potassium phosphate buffer, pH 7.0). After rinsing the column with the equilibration buffer containing 300 mM KCl the pure cDPGS was finally eluted with 50 mM potassium phosphate buffer containing 500 mM KCl at pH 7.0.

3. RESULTS AND DISCUSSION

3.1. *Biosynthesis of cDGP*

Incubation of the crude extract from *Mt. fervidus* with [^{32}P]2-PG in the presence of $MgCl_2$, ATP, and KCl results in the formation of labelled 2,3-DPG and cDPG (Fig. 1A). The same incubation mixture with [2-^{32}P]2,3-DPG instead of 2-PG yields only labelled cDPG (Fig. 1B). These findings indicate that cDPG is formed from 2-PG via 2,3-DPG.

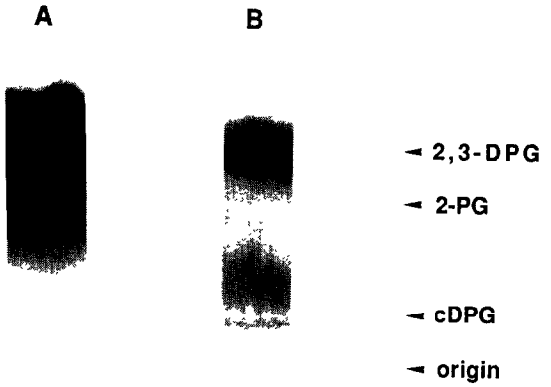

Fig. 1. cDPG biosynthesis with labelled [^{32}P]2-PG (A) and [2-^{32}P]2,3-DPG (B). The reactions were performed under standard assay conditions in the presence of 500 mM KCl with crude extract (25 mg protein/ml) for 30 min. [2-^{32}P]2,3-DPG formed in (A) was used for the labelling experiment in (B). [^{32}P]2-PG was produced with 5 mM pyruvate, 5 mM ATP, 5 mM $MgCl_2$, 5 μCi [γ-^{32}P]ATP, 10 U pyruvate kinase and 10 U enolase at 30°C for 1 h. The labelled compounds were separated on cellulose thin-layer plates. Solvent mixture: 10 mM NH_4CO_3 in 70% methanol.

We confirmed this suggestion by purifying the 2-phosphoglycerate kinase and the cyclic 2,3-diphosphoglycerate synthetase, which are able to catalyze the proposed reactions (see below, Table I, Fig. 2).

Analysis of the resulting nucleotides and inorganic phosphate according to [13,14] demonstrated that the 2PGK forms 1 mol ADP per mol 2,3-DPG from ATP and 2-PG. Thereby 2PGK converts specifically 2-PG and does not accept 3-phosphoglycerate or glycerate.

The cDPGS produces 1 mol ADP and P_i per mol cDPG from ATP and 2,3-DPG. Inorganic phosphate is released from ATP, which is indicated by the formation of labelled cDPG from [3-^{32}P]2,3-DPG and [2-^{32}P]2,3-DPG but not from [γ-^{32}P]ATP (Fig. 3). Thus, ATP serves only as an energy source for the reaction catalyzed by cDPGS.

The reverse reaction from cDPG to 2-PG via 2,3-DPG could not be detected either with crude extract or with the purified enzymes from *Mt. fervidus*.

From these findings we conclude that cDPG is produced by the phosphorylation of 2-PG followed by an intramolecular cyclization of 2,3-DPG.

$$\text{2-PG} + \text{ATP} \xrightarrow{Mg^{2+},\ \text{2PGK}} \text{2,3-DPG} + \text{ADP}$$

$$\text{2,3-DPG} + \text{ATP} \xrightarrow{Mg^{2+},\ \text{cDPGS}} \text{cDPG} + \text{ADP} + P_i$$

The reactions of the cDPG-biosynthesis found for *Mt. fervidus* seem to be generally valid since we detected the activities of 2PGK and cDPGS also in other cDPG-containing methanogens (*Mb. bryantii, Mb. thermoautotrophicum, Methanopyrus spec.*) [15]. We could not confirm the hypothesis of Evans et al.

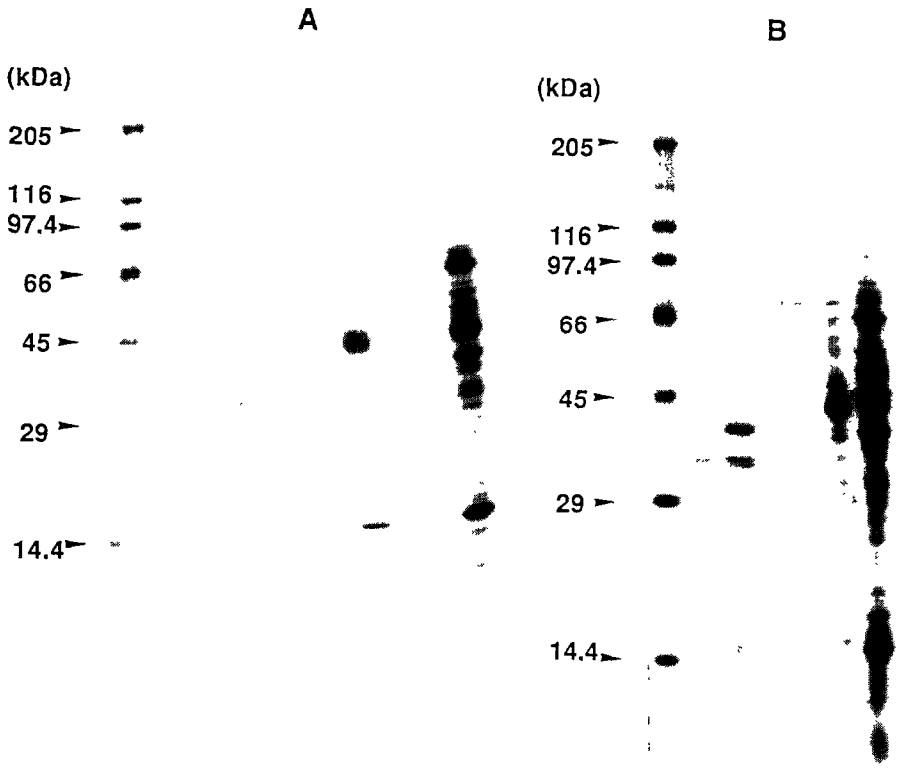

Fig. 2. SDS-PAGE showing the purification steps of 2PGK (A) and cDPGS (B) from *Mt. fervidus*. (A) from right to left: crude extract; hydroxyapatite; ADP-agarose; molecular weight standards. (B) from right to left: crude extract; Q-Sepharose; hydroxyapatite; ATP-agarose; 2,3-DPG-agarose; molecular mass standards. The molecular mass standards contained lysozyme (14.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase *b* (97.4 kDa), β-galactosidase from *E. coli* (116 kDa) and myosin (205 kDa).

[11,12] who postulated that cDPG is directly formed from phospho*enol*pyruvate, and that cDPG represents a precursor of 2,3-DPG. Also their suggestion, that cDPG serves as an obligate intermediate of the gluconeogenesis is unlikely since all gluconeogenetic enzymes were detected in *Mb. thermoautotrophicum* [16,17] and *Mt. fervidus* [18].

From the described reactions of the biosynthesis, cDPG does not seem to represent a storage compound for ATP or phosphate. The 2 mols of ATP consumed by the synthesis of cDPG cannot be recovered because the reverse reactions are not detected. Also the formation of the phosphate anhydride ATP from the phosphate ester 2,3-DPG is thermodynamically unfavourable.

Because inorganic phosphate is released from ATP during the synthesis of cDPG this compound would be inefficient for phosphate storage. Although there is a detectable formation of P_i from cDPG-pool under P_i starvation in *Mb. thermoautotrophicum* [9] cDPG was not accumulated in the stationary phase as would be expected for a storage compound [4].

Table I

Purification of 2PGK and cDPGS from 10 g *Mt. fervidus* cells

Enzyme	Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
2PGK	crude extract	643	18	0.03	–	100
	hydroxyapatite	111	31	0.28	9.3	172
	ADP-agarose	0.05	10	200	6667	56
cDPGS	crude extract	672	14	0.02	–	100
	Q-Sepharose	82	10	0.12	6	71
	hydroxyapatite	13	9	0.7	35	64
	ATP-agarose	1	4	4	200	29
	2,3-DPG-agarose	0.097	2	20.6	1030	14

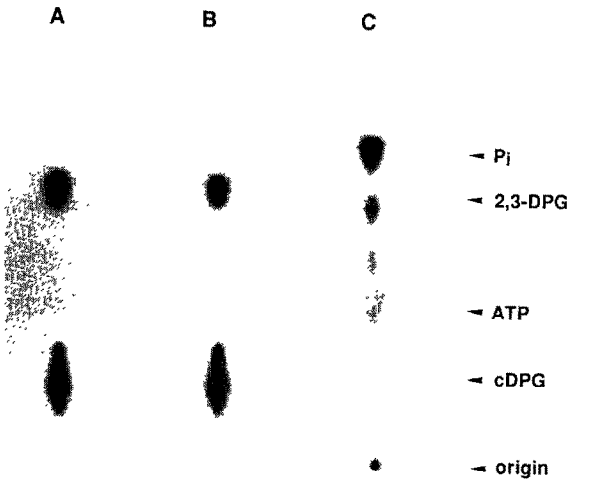

Fig. 3. Intramolecular cyclization of 2,3-DPG with labelled [2-^{32}P]2,3-DPG (A), [3-^{32}P]2,3-DPG (B) and [γ-^{32}P]ATP (C). The reactions were performed with 20 mU of pure cDPGS under standard assay conditions for 1 h. [2-^{32}P]2,3-DPG was formed by the method described in Fig. 1. [3-^{32}P]2,3-DPG was produced with 200 mU of pure 2PGK under standard assay conditions in the presence of 5 μCi [γ-^{32}P]ATP for 30 min. All labelled compounds were separated on cellulose thin-layer plates using the solvent mixture ethyl acetate/acetic acid/water/ammonia (6:6:2:1, v/v).

3.2. *Characterization of 2PGK and cDPGS from* Mt. fervidus

One predominant protein band was found for each prepared enzyme in the SDS-PAGE with an apparent molecular mass of 32.5 kDa for 2PGK and 38 kDa for cDPGS (Fig. 2). The apparent molecular masses of the native enzymes were determined by gel filtration (not shown) of the crude extract from *Mt. fervidus* with standard proteins of known molecular mass. Values of 1400 kDa for 2PGK and 145 kDa for cDPGS were determined indicating that 2PGK represents a homomeric multimer and cDPGS a homomeric tetramer.

The temperature dependence of 2PGK and cDPGS activity was investigated from 30 to 95°C. The respective Arrhenius plots (not shown) demonstrate linearity up to 75°C with relatively low activation energies for both enzymes (Table II). The temperatures of maximal enzyme activities (Table II) are 3–8°C below the optimal growth temperature (83°C) of *Mt. fervidus* probably due to the relatively low intrinsic thermostability of these enzymes. We assume that both enzymes are stabilized by the intracellular milieu similarly to the glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase of this organism [5].

2PGK and cDPGS depend on Mg^{2+} for maximal activity and are almost completely inhibited by 5 mM EDTA under standard assay conditions. We could not estimate the K_d for Mg^{2+} because residual enzyme activities (8% for 2PGK, 4% for cDPGS) remain under essentially 'Mg^{2+}-free' assay conditions with Chelex 100-treated solutions. We assume that Mg^{2+} binds with high affinity to the enzymes.

K^+ activates cDPGS and 2PGK; cDPGS 2.4-fold (maximally activating KCl concentration: 0.5 M) and 2PGK 1.4-fold (maximally activating KCl concentration: 0.3 M). Under physiological K^+ concentrations (700–1000 mM), which were simulated by KCl, cDPGS and 2PGK are activated 2-fold and 1.2-fold, respectively. The activation varies by only 10% for both enzymes in the whole physiological concentration range. NaCl concentrations up to 1 M do not influence the enzyme activities virtually.

The substrate saturation curves of the 2PGK and cDPGS follow the kinetics of Michaelis and Menten [19]. The K_m values of ATP and 2,3-DPG (Table II) are below the intracellular concentrations of ATP (5–7 mM) in *Mb. thermoautotrophicum* [20] and 2,3-DPG (20 mM) in *Mt. fervidus* [18].

A regulation of the cDPG biosynthesis has been presumed from metabolic inhibitor studies [3] and feeding experiments with ^{13}C-precursors [11,12]. From our findings it could not be excluded that the cDPG biosynthesis is partly controlled by the intracellular substrate concentrations. A regulation of the cDPG pool by K^+ was debated [10,12]. Our data indicate that the 2PGK and cDPGS are adapted to rather than regulated by the high intracellular K^+ concentration in *Mt. fervidus*. The only hint of regulation of the cDPG biosynthesis comes from the striking increase of the 2PGK activity after the chromatography on hydroxyapatite, which may be indicative for the presence of a specific inhibitor in the crude extract. cDPG itself does not represent this putative inhibitor because it inhibits the 2PGK only slightly at the physiological concentration of 300 mM (residual activity: 80%).

For understanding the function of cDPG and the regulation of its pool the knowledge of its degradation is necessary. Studies of the cDPG-converting enzymes are currently underway.

Table II

Enzymatic characteristics of 2PGK and cDPGS from *Mt. fervidus*

	2PGK	cDPGS
$T_{max.act.}$ (°C)	77	75–80
E_A (kJ/mol)	44	43
$pH_{opt.}$	6–8	5–7.5
k_{cat} (s^{-1})	108	13
K_m (mM)		
2-PG	0.5	–
ATP	2.7	1.3
2,3-DPG	–	5.5
M_r (kDa)		
Subunit	32.5	38
Native	1400	145

The dependencies of the enzyme activities on temperature and pH were measured with crude extract (22 mg protein/ml). The substrate kinetics were performed with purified enzymes (2 μg protein/ml)

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