Cyclic 2,3-diphosphoglycerate metabolism in *Methanobacterium thermoautotrophicum* (strain ΔH): characterization of the synthetase reaction

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**Abstract** The levels of cyclic 2,3-diphosphoglycerate (cDPG) in methanogenic bacteria are governed by the antagonistic activities of cDPG synthetase and cDPG hydrolase. In this paper we focus on the synthetase from *Methanobacterium thermoautotrophicum*. The cytoplasmic 150kDa enzyme catalyzed cDPG synthesis from 2,3-diphosphoglycerate (apparent \( K_m = 21 \text{ mM} \)), Mg\(^{2+} \) (\( K_m = 3.1 \text{ mM} \)) and ATP (\( K_m = 1-2 \text{ mM} \)). In batch-fed cultures, the enzyme was constitutively present (6-6.5nmol per min per mg protein) during the different growth phases. In continuous cultures, activity decreased in response to phosphate limitation. The synthetase reaction proceeded with maximal rate at pH6 and at 65°C and was specifically dependent on high (> 0.3 M) K\(^+ \) concentrations. The reaction conditions remarkably contrasted to those of cDPG degradation catalyzed by the previously described membrane-bound cDPG hydrolase.

**Key words** *Methanobacterium thermoautotrophicum* Cyclic 2,3-diphosphoglycerate · 2,3-Diphosphoglycerate Cyclic 2,3-diphosphoglycerate synthetase

**Abbreviations** cDPG Cyclic 2,3-diphosphoglycerate 2,3-DPG 2,3-Diphosphoglycerate · 2-PG 2-Phosphoglycerate · 3-PG 3-Phosphoglycerate

**Introduction**

Cyclic 2,3-diphosphoglycerate (cDPG), a 2,3-diphosphoglycerate derivative in which both phosphate groups are linked by a pyrophosphate bond (Kanodia and Roberts 1983; Seeley and Fahrney 1983, 1984a), is a cell constituent of some methanogenic species. In these organisms, cDPG concentrations vary between 1 mM and 1 M (Tolman et al. 1986; Hensel and König 1988; Huber et al. 1989; Gorris et al. 1990; Rudnick et al. 1990). The metabolic function of cDPG has not been unequivocally established and it has been suggested that cDPG acts as a storage of energy (Gorris et al. 1990; Van Alebeek et al. 1991), phosphorous (Seeley and Fahrney 1983), or cell carbon (Seeley and Fahrney 1984b; Evans et al. 1985). Furthermore, cDPG as the counter ion of K\(^+ \) protects enzymes against heat inactivation (Hensel and König 1988).

Biosynthesis and degradation of cDPG has been studied in two methanogens, *Methanobacterium thermoautotrophicum* (strain ΔH) and *Methanothermus fervidus*, and is connected with the gluconeogenic route as proposed in Fig. 1 (Lehmacher et al. 1990; Van Alebeek et al. 1991, 1992, 1994). In addition, in *M. thermoautotrophicum* cDPG appears to be the precursor of some polymers (Gorovkenko and Roberts 1993). In *M. thermoautotrophicum*, cDPG degradation occurs by hydrolysis of the pyrophosphate bond (Sastry et al. 1992; Van Alebeek et al. 1994), followed by dephosphorylation of 2,3-DPG into 3-phosphoglycerate (3-PG) (Van Alebeek et al. 1991). In *M. fervidus*, cDPG is synthesized from 2-phosphoglycerate.
(2-PG) in two subsequent ATP-dependent reactions. The enzymes involved, 2-phosphoglycerate kinase (2-PG kinase) and cyclic 2,3-diphosphoglycerate synthetase (cDPG synthetase), have been purified and characterized to some extent (Lehmacher et al. 1990). In *M. thermoautotrophicum*, only the latter reaction has been briefly described (Van Alebeek et al. 1992).

At the levels of 2- and 3-phosphoglycerate (phosphoglycerate mutase) and 2,3-DPG, the routes of cDPG biosynthesis and degradation are interconnected (Fig. 1). Uncontrolled metabolism could then give rise to the futile use of ATP. Hence, one might expect cDPG and 2,3-DPG synthesis and hydrolysis to be subject to some form of regulation. Since nothing is specifically known about this regulation, it was of interest to investigate cDPG synthetase activity at the level of protein synthesis. cDPG synthetase activity was determined in batch-fed and continuous cultures. The results are discussed in view of the proposed scheme for cDPG metabolism (Fig. 1; Van Alebeek et al. 1991).

**Materials and methods**

**Materials**

Procion Green HE-4BD (ICI; Manchester, UK) was a gift from Dr. J. Visser, Agricultural University of Wageningen, The Netherlands. The dye was immobilized [3 g dye/25 g Sepharose CL-6B (Pharmacia)] as documented previously (Hondman and Visser 1990). All other chemicals were of highest grade available.

**Organism, culturing and preparation of extract**

*M. thermoautotrophicum* strain AH (DSM 1053) was cultured at 65°C under H$_2$-CO$_2$ (80:20, v/v) in a defined medium (Gorris et al. 1990); Na$_2$S was replaced by NaS$_2$O$_3$ (4 mM). For growth experiments, cells were cultured either in a 0.5-L continuous fermentor (D, 0.173 h$^{-1}$; culture volume, 258 ml; flow rate, 1 ml/min) or in a 10-L batch fermentor (OD$_{578}$ as the amount of enzyme synthesizing 1 nmol cDPG per min).

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**Enzyme assays**

The standard reaction mixtures (200 ml) prepared at ambient temperature in 10-ml serum bottles contained 40 mM MgCl$_2$, 20 mM ATP, 0.5 M KCl, 20 mM 2,3-DPG, 50 mM TES/K$^+$ buffer (pH 6 at 60°C), and enzyme fractions as indicated. The reactions were carried out under air were started by placing the vials at 60°C. After an appropriate incubation period, the reactions were stopped by placing the vials on ice. Subsequent Samples were prepared and analyzed by HPLC with 0.5 mM oxalate as the internal standard as described previously (Van Alebeek et al. 1992, 1994). cDPG synthetase activity was determined on the basis of cDPG increase, or 2,3-DPG decrease. An activity of 1 mU is defined as the amount of enzyme synthesizing 1 nmol cDPG per min.

**Results**

**cDPG synthetase activity in Methanobacterium thermoautotrophicum**

Previously, we reported the presence of cDPG synthetase in *M. thermoautotrophicum* and found activities of about 3 mU per mg crude extract resolved from low-molecular-weight compounds (cofactor-free extract; Van Alebeek et al. 1992). Here, we followed synthetase activities in the organism during growth in batch-fed (Fig. 2) and continuous (Table 1) fermentors.

**Growth of *M. thermoautotrophicum***

In the batch fermentor was characterized by a short exponential phase followed by an extended period during which cell mass linearly increased. During this phase, cell growth is limited by the hydrogen supply (Seely and Fahrney 1984b;