Cloning, Sequencing, and Expression of the Gene Encoding Cyclic 2,3-Diphosphoglycerate Synthetase, the Key Enzyme of Cyclic 2,3-Diphosphoglycerate Metabolism in *Methanothermus fervidus*

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Cyclic 2,3-diphosphoglycerate synthetase (cDPGS) catalyzes the synthesis of cyclic 2,3-diphosphoglycerate (cDPG) by formation of an intramolecular phosphoanhydride bond in 2,3-diphosphoglycerate. cDPG is known to be accumulated to high intracellular concentrations (>300 mM) as a putative thermoadapter in some hyperthermophilic methanogens. For the first time, we have purified active cDPGS from a methanogen, the hyperthermophilic archaean *Methanothermus fervidus*, sequenced the coding gene, and expressed it in *Escherichia coli*. cDPGS purification resulted in enzyme preparations containing two isoforms differing in their electrophoretic mobility under denaturing conditions. Since both polypeptides showed the same N-terminal amino acid sequence and Southern analyses indicate the presence of only one gene coding for cDPGS in *M. fervidus*, the two polypeptides originate from the same gene but differ by a not yet identified modification. The native cDPGS represents a dimer with an apparent molecular mass of 112 kDa and catalyzes the reversible formation of the intramolecular phosphoanhydride bond at the expense of ATP. The enzyme shows a clear preference for the synthetic reaction: the substrate affinity and the $V_{\text{max}}$ of the synthetic reaction are a factor of 8 to 10 higher than the corresponding values for the reverse reaction. Comparison with the kinetic properties of the electrophoretically homogeneous, apparently unmodified recombinant enzyme from *E. coli* revealed a twofold-higher $V_{\text{max}}$ of the enzyme from *M. fervidus* in the synthesizing direction.

Reports on the high intracellular concentrations of low-molecular-weight compounds in prokaryotes (members of the *Bacteria* as well as the *Archaea*) adapted to high temperatures have become more common over the last few years, leading to speculation about a role for these compounds as thermoadaptors. In some cases, the thermoadaptive function is supported by the observed dependence of the intracellular concentrations of the compounds on growth temperature, as found for cyclic 2,3-diphosphoglycerate (cDPG), di-my-o-inositol-1,1'-phosphate, and 2-O-β-d-mannosyl-di-my-o-inositol-1,1'-phosphate (8, 15, 26, 27). In line with the proposed thermoadaptive function, some of these compounds such as cDPG, di-my-o-inositol-1,1'-phosphate, and mannosylglycerate stabilize proteins against heat inactivation in vitro (15, 32, 41). However, for a reliable assessment of the physiological function of these compounds, studies on their individual interactions with various cell constituents and detailed analyses of the regulation of their intracellular concentration in response to environmental factors are needed, and these have not been performed.

The first low-molecular-weight compound found at conspicuously high concentrations in thermophiles is the trianionic 2,3-diphosphoglycerate (cDPG), whose highest concentrations (>300 mM) have been observed in the hyperthermophilic methanogens *Methanothermus fervidus*, *Methanobacterium sociabilis*, and *Methanopyrus kandleri* (15, 21). The compound was first described for *Methanobacterium thermoautotrophicum* (17, 42), and its occurrence seems to be restricted to certain methanogenic members of the *Archaea* (members of *Methanobacteriales*, *Methanomicrobiales*, and *Methanopyrales*) (13, 15, 21, 23, 35, 46).

The metabolism of this unusual compound has been studied mainly in the moderately thermophilic *Methanobacterium thermoautotrophicum* and the hyperthermophilic *Methanothermus fervidus*. From enzymatic analyses, Lehmacher et al. (24) proposed that the biosynthesis of cDPG in *M. fervidus* branches off the main carbon metabolism at 2-phosphoglycerate (2-PG) and proceeds via two ATP-dependent reactions catalyzed by 2-phosphoglycerate kinase (2-PGK) and cyclic 2,3-diphosphoglycerate synthetase (cDPGS). In vivo nuclear magnetic resonance spectroscopy studies with *M. thermoautotrophicum* (12), as well as comparative enzymatic analyses with cell extracts of various cDPG-containing methanogens (23), indicate that the proposed biosynthetic pathway applies in general. For the degradation of cDPG, data are available only for *M. thermoautotrophicum*. Pulse-chase experiments and enzymatic investigations indicated a degradation pathway via 2,3-diphosphoglycerate (DPG) by cDPG hydrolase activity (40, 49). Finally, a DPG phosphatase converts DPG back to 2-PG, as described by Van Alebeek et al. (47).

To complete our knowledge about the mechanism and regulation of the cDPG metabolism in hyperthermophilic methanogens and to address the physiological role of cDPG in these hyperthermophiles, we have focused on the enzymes involved in the biosynthesis and breakdown of cDPG in *M. fervidus* and on their regulation in response to growth parameters. To date, only the 2-PGK from *M. fervidus* has been amenable to purification and isolation in sufficient yields. Its coding gene was cloned, sequenced, and expressed in *Escherichia coli*, providing
the basis for detailed transcriptional analyses and biochemical studies (25). In contrast, cDPGS, which catalyzes the unusual formation of the intramolecular pyrophosphate bond in DPG, resisted proper enzyme characterization due to its high lability toward oxygen and its low ionic strength. Here we describe a more efficient purification procedure for the enzyme than that given by Lehmacher et al. (24), which allows a more reliable characterization of the enzyme protein, and we report on the cloning, sequencing, and overexpression of the coding gene in E. coli. The functional and structural properties of the enzyme are interpreted with respect to regulation of the intracellular DPG pool in M. fervidus.

MATERIALS AND METHODS

Materials. DPG and acid phosphatase were purchased from Sigma. The test kit for DPG was from Boehringer Mannheim. Media for column chromatography were from Pharmacia (Q-Sepharose FF, Superdex 16/200 prepgrade, and phenyl-Sepharose), Fluka (hydroxyapatite #8), or Sigma ( Reactive Green 19 agarose). All other chemicals (analytical grade) were from Fluka, Gerbu, Sigma, or Boehringer Mannheim.

Plasmids. PCR products and restriction fragments were cloned by using plasmids pGEM-3Z(f+) (Promega) and pBluescript II KS+(+) (Stratagene), respectively. For heterologous expression, the vector pF118EHE was used (10).

Bacterial strains and growth conditions. Mass cultures of M. fervidis DSM 2088 were grown to the late logarithmic phase as described previously (16). For cloning and expression experiments, E. coli XL1Blue (Stratagene) and DH5α (Life Technologies) were grown under standard conditions.

Protein determination, N-terminal sequencing, and SDS-PAGE. The protein content was determined by the method of Bradford (4) with the Bio-Rad test kit (Boehringer Mannheim). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 7.5 or 10% polyacrylamide gels (22) were used. The protein solutions were incubated at 95°C for 1 min in sample buffer (22) containing 5% mercaptoethanol prior to electrophoresis.

Enzyme assay and determination of kinetic parameters. The enzyme activity in the biosynthetic and hydrolytic direction was determined as described previously (24) but with the Bio-Rad test kit and bovine serum albumin as the standard. Protein sequencing was performed by automated Edman degradation with a gas phase sequencer (Applied Biosystems). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 7.5 or 10% polyacrylamide gels (22) were used. The protein solutions were incubated at 95°C for 1 min in sample buffer (22) containing 5% mercaptoethanol prior to electrophoresis.

Determination of the molecular mass of native cDPGS. Mass determinations were performed on a Superdex 16/200 prepgrade column equilibrated with 10 mM TES/KOH (pH 7.0) containing 1 mM di-thio-dt, DTT and (875 mM KCl at 83°C and under anaerobic conditions. The apparent Kₘ and Vₘₐₓ values were deduced from transformation plots of saturation curves by the method of Hanes (14).

Test for glycosylation. The Bio-Rad Immun-Blot kit was used to detect glycosylated proteins separated by SDS-PAGE.

Electrospray mass spectrometry. The protein solution was applied to a reversed-phase high-pressure liquid chromatography column coupled on-line to an atmospheric pressure ionization source fitted to an API III tandem quadrupole (Boehringer Mannheim). Genomic DNA was transferred to Biodyne B nylon membranes ( Pall) by capillary blotting (6). Southern blots were hybridized at 20°C with 5 SSC (1× SSC is 0.15 M NaCl plus 0.1 M sodium citrate) (probe 1) or at 26°C with 5× SSC (probe 2) and washed at 30°C with 0.1× SSC or at 41°C with 0.5× SSC, respectively. A 2.8-kb DraI fragment giving a strong hybridization signal was selected, cloned, and sequenced with an automated laser fluorescent DNA sequencer (Pharmacia) (36, 38, 51). The sequence was determined in both directions.

Expression of the cpgS gene from M. fervidus in E. coli. For expression of the cpgS gene in E. coli, two new restriction sites (MunI and BamHI) were intro-
duced into the flanking regions of the cpgS gene by PCR mutagenesis with the oligonucleotide primers (new restriction sites are underlined) 5′GGTAAGGGGGCGGACATTGATGGTTAAGACGGAAC5′ and 5′TATTGGACCCCTTAGATTAATTTCAAGTCATGGG3′. PCR amplification was performed with the Expand High-Fidelity PCR system (Boehringer Mannheim) as specified by the manufacturer. The PCR product was digested with the respective restriction enzymes and cloned into the expression vector pF118EHE. The gene sequence was confirmed on both strands. Since N-terminal sequencing of the recombinant cDPGS (rDPGS) revealed incomplete processing of the N-terminal methionyl residue, the methionine aminopeptidase gene (map) of E. coli (3) was cloned directly downstream of the cpgS gene by using the restriction sites BamHI and HindIII. Restrictive restriction sites were introduced upstream (BamHI) and downstream (HindIII) of the gene by PCR with the mutagenic primers 5′TAATTTTGCGGATCCCGGC5′ and 5′GGGTGTTGAGCGATCCCGCG5′. A 2.8-kb DraI fragment giving a strong hybridization signal was selected, cloned, and sequenced with an automated laser fluorescent DNA sequencer (Pharmacia). For expression, the method of Maekin et al. (29). The gene encoding cDPGS was identified by hybridization with two oligonucleotide probes (probe 1, 5′GARACNAARAARATGAT3′; probe 2, 5′GGAYGGNGAARCAYTTYCC3′) derived from two regions of the N-ter-
iminoase aminopeptidase of E. coli; rrrB, terminator-fragment containing of the rrrB operon of E. coli; bla, gene encoding b-lactamase of E. coli; lacZ, gene coding for the Lac repressor of E. coli.

FIG. 1. Physical map of the recombinant expression vector pFj cDPGS. cpgS, gene coding for the cDPGS of M. fervidus; map, gene coding for methio-

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centrifugation at 40,000 \times g at 4°C, the supernatant was incubated for 30 min at 75°C, cooled on ice, and centrifuged again. Subsequently, the protein solution was diluted 1:1 (vol/vol) with buffer A (without KCl but in the presence of 10 mM DTT), applied to the reactive dye column (0.8 by 7 cm) and separated by the same elution program as described for the enzyme from \( M. fervidus \).

Sequence analysis and computer alignments. For sequence analyses, the program GENMON, version 4.4 (GBF Braunschweig), was used. Homology searches were performed with BLASTP and BLASTX (1). The source of sequence information was GenBank (update August 1998).

Nucleotide sequence accession number. The nucleotide sequence has been deposited at the EMBL Nucleotide Database under accession number Y09856.

RESULTS

Purification of cDPGS from \( M. fervidus \). In a previous paper (24), the biosynthetic pathway of cDPG has been analyzed and the two enzymes involved in the biosynthesis, 2-PGK and cDPGS, have been described. Whereas the 2-PGK catalyzing the phosphorylation of 2-PG could be isolated in satisfactory yield, the cDPGS catalyzing the ring formation through an anhydride bond between the phosphate groups of DPG proved to be very labile, especially in the enriched state, impeding a successful preparation for structural and functional analyses. In this study, an improved purification procedure, taking into account the high lability of the enzyme at low ionic strength and its high susceptibility toward oxidation, is elaborated.

The purification of the enzyme comprising three chromatographic steps (chromatography on phenyl-Sepharose, hydroxyapatite ff, and Reactive Green 19 agarose) is documented in Fig. 2 and Table 1. Remarkably, the purified fractions contain two polypeptides with apparent molecular masses of 57 and 59 kDa, as deduced from SDS-PAGE (Fig. 2), thus differing from the previous report, which affiliated the cDPGS activity to a protein with a subunit mass of 38 kDa (24). The relative intensity of both protein bands (after staining the gels either with Coomassie blue or AgNO\(_3\)) [2] was not changed by applying different purification conditions. Further attempts to separate the protein species under native conditions by using metal-chelating chromatography and size exclusion chromatography failed.

As revealed by Edman degradation, the N termini of both polypeptides resolved by SDS-PAGE showed the same sequence (GETKRMIXLVDGEHYFPVVK). This finding, together with Southern experiments yielding only one positive signal with the restricted genomic DNA of \( M. fervidus \) (see below), indicates that both protein species represent isoforms encoded by the same gene but differing from one another with respect to conformation or chemical modification. As deduced from the yield of the first residue cleaved off in the course of Edman degradation (11.7 or 11.4 pmol, respectively), the protein contents in the two bands were equivalent, confirming the visual impression of the stained gels.

Quaternary structure of cDPGS. The apparent molecular mass of cDPGS was determined by size exclusion chromatography to be 112 kDa under native conditions. Considering the apparent molecular mass of both polypeptides resolved by SDS-PAGE (57 and 59 kDa), cDPGS apparently represents a dimer in its native state. At present, we cannot decide whether cDPGS from \( M. fervidus \) is a heterodimer composed of two isomeric subunits or represents a 1:1 mixture of two homomeric dimers differing in conformation or chemical modification.

To detect mass differences, which may be indicative of the nature of the modification, mass determinations were performed with the enzyme preparation by using electrospray mass spectrometry. However, the measurements resulted only in one main molecular mass of 50,663 ± 6 Da, close to the theoretical mass of 50,657.1 calculated from the deduced amino acid sequence (see below). The mass difference between the two forms may be too small to be resolved by this method, or the two forms may represent different conformers of the same cofunctional structure. Attempts to establish chemical modifications failed, however. Thus, incubation of the protein in sample buffer (1 to 5 min at 95°C) containing up to 10 mM DTT or 25% 2-mercaptoethanol (instead of 5% 2-mercaptoethanol) to cleave barely accessible disulfide bridges did not change the electrophoretic pattern. Furthermore, treatment with phosphatase or phosphodiesterase did not give hints about modifications. Also, incubation at 55°C for 2 h at pH 0.5 (0.2 M HCl) or pH 11 (2 M NH\(_4\)OH) or in the presence of 0.2 M hydroxylamine (adjusted with succinic acid to pH 3.5) to cleave off putative phosphoryl groups linked to various resi-

<table>
<thead>
<tr>
<th>Purification step</th>
<th>( M. fervidus )</th>
<th>( E. coli )</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total amt of protein (mg)</td>
<td>Total activity (U)</td>
</tr>
<tr>
<td>Crude extract</td>
<td>580</td>
<td>10.0</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>18.0</td>
<td>12.3</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.75</td>
<td>3.1</td>
</tr>
<tr>
<td>Reactive Green 19 agarose</td>
<td>0.07</td>
<td>2.0</td>
</tr>
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</table>
sequence of ORF Y, no similarity to any known protein could be orientation), are truncated. For the deduced amino acid se-
cpgS two other reading frames, ORF Y (downstream of cpgS gene is completely contained within the Dra II fragment giving positive hybridization signals with Dra II, indicating the pres-
duities was not successful. Specific labeling for glycoproteins after separation by SDS-PAGE yielded negative results.

To investigate whether the different electrophoretic mobilities of the two polypeptides are due to differences in conformation without covalent modifications being involved, electrophoresis was performed with cDPGS pre-denatured with guanidinium hydrochloride. For this purpose, the protein was incubated in 10 M guanidinium hydrochloride at 90°C in the presence of 10 mM DTT for 2 h and subsequently dialyzed against 50 mM TES buffer (pH 7.0) containing 250 mM KCl and 10 mM DTT to remove the denaturant. Also, after this treatment, which resulted in complete loss of activity and in precipitation of the protein, the electrophoretic pattern did not change, suggesting that the observed differences in electrophoretic mobility are not due to conformational differences.

**Catalytic properties of cDPGS from** **M. fervidus.** In contrast to previous results, which characterized the cDPGS from *M. fervidus* as a unidirectional enzyme that exclusively catalyzes the synthesis of cDPG (24), the present data clearly show that the enzyme also catalyzes the hydrolysis of cDPG but with significantly lower rates and lower affinities for the respective substrates (Table 2). Hydrolysis of cDPG could be observed only in the presence of ADP and P_i, confirming the real reversibility of the enzyme reaction. The inability to monitor the reverse reaction in earlier experiments may be explained by the use of HEPES as the assay buffer (instead of TES, which was used in recent experiments), which proved to inhibit the reverse reaction strongly. The kinetic parameters *K_m* and *V_max* for 2,3-DPG, cDPG, ADP, and ATP were determined at the optimal growth temperature of 83°C in the presence of 875 mM KCl, to mimic physiological reaction conditions with respect to temperature and intracellular ion concentration (15).

**Nucleotide sequence of the cpgS gene and its flanking regions.** Southern hybridization analyses with two degenerated oligonucleotide probes deduced from the N-terminal amino acid sequence of the cDPGS (see Materials and Methods) gave only a single signal with the genomic DNA of *M. fervidus* digested with EcoRI, XbaI, PstI, and DraII, indicating the presence of only one gene coding for cDPGS. Sequencing of a 2.8-kb DraII fragment giving positive hybridization signals with both probes revealed three open reading frames (ORFs) (Fig. 3). One of them was identified as the cpgS gene by correspondence of the deduced N-terminal amino acid sequence and the determined sequence of the purified protein. While the cpgS gene is completely contained within the DraII fragment, the two other reading frames, ORF Y (downstream of cpgS, same orientation as cpgS) and ORF X (upstream of cpgS, opposite orientation), are truncated. For the deduced amino acid sequence of ORF Y, no similarity to any known protein could be found, whereas a database search revealed significant similarity of the deduced amino acid sequence of ORF X to dihydroxy-acid dehydratases from members of the *Bacteria* (58.1 and 59.5% identity to the enzymes from *Clostridium pasteurianum* or *E. coli*, respectively) and *Eucarya* (44.6% identity to the *Saccharomyces cerevisiae* enzyme).

The coding sequence of the cpgS gene encompasses 1,380 bp (start codon, ATG; stop codon, TGA) (Fig. 4). The G+C content of the gene has been calculated to be 32.8%, which is in accordance with the average genomic G+C content of *M. fervidus* (33% [45]). At 6 bp upstream of the cpgS gene, a putative ribosome-binding site, which corresponds exactly to the complementary sequence at the 3′ end of the 16S rRNA of *M. fervidus*, could be identified (34). The upstream region contains also sequence motifs resembling consensus sequences established for Box A and B of the archaeal promoter (CTT AAC [positions −52 to −47] and ATAC [positions −24 to −21]), suggesting that these sequences function as start signals for transcription (33). No indications for the presence of transcription termination signals downstream of the cpgS gene could be obtained. As a possible explanation, ORF Y and the cpgS gene are cotranscribed.

**Characteristics of the deduced amino acid sequence of the cDPGS of** **M. fervidus.** The reading frame codes for 460 residues. Without the N-terminal methionyl residue processed in *M. fervidus* (shown by sequencing the mature protein), the theoretical molecular mass was calculated to be 50,657.1 Da, taking the average isotope heterogeneity into account. The significantly higher masses determined by SDS-PAGE (57,000

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### Table 2. Kinetic parameters of the cDPGS from *M. fervidus* compared with those of the enzyme produced in transformed *E. coli*<sup>a</sup>

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Substrate</th>
<th><em>M. fervidus</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>K_m</em> (mM)</td>
<td><em>V_max</em> (U/mg)</td>
<td><em>K_m</em> (mM)</td>
</tr>
<tr>
<td>cDPG synthesis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ATP</td>
<td>3.0 ± 1.5</td>
<td>3.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>DPG</td>
<td>5.8 ± 2</td>
<td>32.0 ± 4</td>
</tr>
<tr>
<td>cDPG hydrolysis&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ADP</td>
<td>4.7 ± 1.5</td>
<td>4.1 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>cDPG</td>
<td>41.2 ± 4</td>
<td>3.8 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Assays conditions: 10 mM TES/KCl (pH 7.0)–875 mM KCl–5 mM DTT at 83°C. A total of five to seven replicate determinations were used.

<sup>b</sup> Concentration of the fixed substrates: 15 mM ATP and 40 mM DPG.

<sup>c</sup> Concentration of the fixed substrates: 17 mM ADP, 100 mM cDPG, and 50 mM P_i.

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**FIG. 3.** Restriction map and sequencing strategy of the cloned DraII fragment carrying the cpgS gene of *M. fervidus*. The coding regions are marked by bold arrows. Thin arrows indicate the individual sequence runs.
and 59,000 Da) might be due to unusual unfolding in the presence of SDS. A computer search with the M. fervidus cDPGS amino acid sequence on the basis of a GenBank update of August 1998, including the sequence information of the complete genomes of the archaea Methanococcus jannaschii (5), Archaeoglobus fulgidus (19), Methanobacterium thermoautotrophicum (44) and Pyrococcus horikoshii (18), showed significant similarity to ORFs of the genomes of last two species (73 and 42% identity, respectively). Some resemblance to ATP-binding proteins was detected with respect to a sequence motif indicative of phosphate-binding function (the consensus sequence of the so-called phosphate-binding loop is GXXXXGKT [39]); the sequence fragment TGKRIGKT (positions 143 to 150 of the cDPGS sequence) shows preferred similarity to the β-subunits of ATP synthases (the sequence motif of the phosphate-binding loop is GGAGVGKT [39]). The assumption that the respective sequence region may be involved in phosphate binding is supported by secondary-structure predictions (7, 11), which assigned the sequence region to a loop structure flanked by a β-strand and an α-helix in accordance with the topology of ATP-binding proteins.

Expression of the cpgS gene of M. fervidus in E. coli and characterization of the gene product. For heterologous expression, the cpgS gene was amplified by PCR and cloned into the expression vector pJF118EH under control of the tac promoter. N-terminal sequencing of the rcDPGS purified by heat treatment and chromatography on Reactive Green 19 agarose showed that the start methionyl residue is only partially cleaved off. Virtually complete processing could be performed by cloning the E. coli map gene directly downstream of cpgS on the expression plasmid (Fig. 1), using the same strategy as described by Sandman et al. (37). Purified enzymes from both preparations showed virtually the same specific activity (14.2 U/mg [partially processed rcDPGS] and 16 U/mg [completely processed rcDPGS], determined for the synthesizing direction). For comparisons of the macromolecular and enzymic properties of the processed rcDPGS and the enzyme isolated from M. fervidus, the enzyme was isolated by the same purification procedures.
method as described above for the enzyme from *M. fervidus*. In this procedure, 0.6 mg of homogeneous enzyme protein could be obtained from 10 g of wet *E. coli* cells. The abbreviated, two-step purification procedure comprising heat treatment (30 min at 75°C) and subsequent chromatography on Reactive Green 19 agarose allowed a threefold-higher recovery.

As documented in Fig. 2, SDS-PAGE of the rcDPGS showed only a single band with an apparent molecular mass of 57 kDa, corresponding to the faster-migrating protein species in enzyme preparations from *M. fervidus*. Preparations from aerobically and anaerobically grown *E. coli* cultures gave the same result. The molecular mass determination by electrospray mass spectrometry yielded a value of 50,655.4 Da, which corresponds to both the mass determined for cDPGS from the original organism (50,663 ± 6 Da) and the value calculated from the deduced amino acid sequence (50,657.1 Da). Like the cDPGS isolated from *M. fervidus*, the native rcDPGS exhibited an apparent molecular mass of 112 kDa, determined by size exclusion chromatography, also accounting for a dimeric association of the recombinant enzyme.

However, when the enzymatic properties were compared rcDPGS differed from the enzyme isolated from the original organism: the maximal activity for cDPGS synthesis of the enzyme produced in *E. coli* amounted to only 50% of that determined for the enzyme isolated from *M. fervidus* (16 U/mg [Table 2]). However, the *V*_max for the hydrolysis reaction and the *K*_m values for all variable substrates tested are identical within the margins of error.

**DISCUSSION**

In the present study, the enzyme which catalyzes the transformation of DPG to cDPG, an unusual DPG derivative previously found exclusively in some members of the methanogenic *Archaea*, was identified. Because of the novelty of the catalyzed reaction and the key role of the enzyme in DPG metabolism, the enzyme deserves special attention for both its mechanistic and physiological aspects. Thus, the unusual formation of an intramolecular phosphoanhydride bond resulting in a seven-membered ring poses special challenges for mechanistic analyses of the ATP-mediated activation of the vicinal phosphate groups for ring closure. Furthermore, from its key role in cDPG metabolism, an adequate regulatory function can be expected to govern the intracellular cDPG concentration by extracellular and/or intracellular signals, which in turn could give indications about the physiological meaning of the striking cDPG accumulation in hyperthermophilic methanogens.

Former attempts to purify the protein responsible for the cDPG-synthesizing activity failed mainly due to the high lability of the enzyme. Thus, the high susceptibility of cDPGS of *M. fervidus* and *M. thermoautotrophicum* to oxidation and inactivation at low ionic strength impeded preparation of pure enzyme (48) or even led to an erroneous identification of the protein (24). With an improved isolation procedure, we are now able to purify the protein from *M. fervidus* in satisfactory amounts, providing the basis for functional and structural studies.

cDPGS of *M. fervidus* showed sequence similarity to the deduced amino acid sequence of an ORF in the genome of the cDPG-positive methanogen *M. thermoautotrophicum* (44), with 73% sequence identity. As expected from the equivalent cDPG synthesis pathway, the *M. thermoautotrophicum* genome also contains an ORF coding for a protein homologous to 2-PGK of *M. fervidus*, exhibiting only a slightly lower similarity score (70% identity) than that found for the cDPGS. Obviously, the genome of *P. horikoshii* also harbors homologous genes coding for 2-PGK and cDPGS (42% identity each), suggesting that members of the *Euryarchaeota* other than methanogens are able to produce cDPG. As a confirmation, cDPG could be detected in *P. woesei*, a close relative of *P. horikoshii*, but at lower concentrations (0.2 μmol/mg of protein [30]). On the other hand, the cDPG-negative methanogen *M. jannaschii* contains a homologue of 2-pgk (43% identity of the deduced amino acid sequence) but is lacking cpgS, suggesting that only cDPGS plays a specific role in the cDPG metabolism. The function of 2-PGK might not be confined to the synthesis of cDPG but may serve additional, hitherto unknown purposes.

As further confirmation of a rather facultative functional coupling of cDPGS and 2-PGK in *M. fervidus* (25), *M. thermoautotrophicum* (44), and *P. horikoshii* (18), the coding genes of the enzymes are not linked in an operon or operon-like structure. Thus, only the reactions leading to closure of the phosphoanhydride bond in DPG and its hydrolysis are specific for cDPG formation or breakdown and therefore seem to be suitable control points for an immediate regulation of the intracellular cDPG pool.

Despite the overall coincidence in the metabolic cDPG pathway, the enzyme reactions responsible for the specific formation and breakdown of cDPG differ in *M. fervidus* and *M. thermoautotrophicum* not only with respect to mechanism but also with respect to regulation, suggesting different physiological roles of cDPG in the two organisms.

In *M. thermoautotrophicum*, formation of the intramolecular phosphoanhydride bond in DPG is catalyzed by an irreversible working ATP-dependent cDPGS activated by K⁺ (49), whereas for the cleavage of the pyrophosphate bond at least two hydrolases, differing in their biochemical and regulatory properties, are responsible; these are a soluble enzyme stimulated by K⁺, Mg²⁺, and DTT but inhibited by DPG (40) and a membrane-bound hydrolase strongly inhibited by P_i and K⁺ (49). For the soluble cDPG hydrolase, an anabolic function has been proposed involving supplying the cell with 3-PG deduced from the cDPG pool for gluconeogenesis (40), whereas the membrane-bound enzyme seems to play a major role in phosphate supply (49): its strong inhibition by P_i and the counterion K⁺, together with the K⁺-induced activation of cDPGS, could be the major cause of the observed increase of the intracellular cDPG level when excess P_i is present in the growth medium (20). Thus, it seems conceivable that cDPG fulfills mainly a storage function for carbon and/or phosphate in *M. thermoautotrophicum*. Since cDPG does not represent the major anion in this organism, possible osmotic stress accompanied with change of intracellular cDPG concentration could be compensated by the dominant anions glutamate and/or 1,3,4,6-tetranetetrahydroxyxylic acid (13). A function of cDPG in energy storage in *M. thermoautotrophicum* seems to be less probable, since neither the hydrolysis of cDPG to DPG nor the dephosphorylation of DPG leading the carbon skeleton of cDPG back to the central carbon metabolism is coupled to ATP recovery. Also, a function of cDPG as a thermoadapter in this organism does not seem very likely, since an increase in growth temperature is not followed by a discernible increase in cDPG levels (13).

In contrast, in *M. fervidus* both the synthesizing and hydrolyzing reactions are catalyzed by the reversibly working cDPGS. At present, no indications of the presence of a cDPG hydrolyase activity in this organism can be observed. Thus, unlike in *M. thermoautotrophicum*, the hydrolytic reaction is accompanied by recovery of 1 mol of ATP in *M. fervidus*. Therefore, cDPG would be more suitable as an energy storage compound for *M. fervidus* than for *M. thermoautotrophicum*. As a further consequence of the reversible reaction of cDPGS, the hydrolytic reaction depends on phosphate and is not inhibited.
by up to 100 mM P₃. As such, the cDPG pool does not seem to be regulated by P₃, and therefore we tend to exclude the role of cDPG as a phosphate storage compound in M. fervidus. Only preliminary information could be obtained about the regulatory properties of cDPGS of M. fervidus. The classical Michaelis-Menten kinetics observed with substrates and cosubstrates exclude homotropic effects. Potassium ions do not play a significant role in regulating the activity of the enzyme within the physiological concentration range (700 to 1,000 mM) (24). Instead, M. fervidus cDPGS seems to be affected by some kind of modification specific for the M. fervidus system, as indicated by the appearance of a second, electrophoretically distinct isoform, which does not occur under the expression conditions of E. coli (in aerobically or acrobically grown cells). Since this isoform cannot be made to revert under strong unfolding conditions (e.g., by incubation with 10 M guanidinium chloride under reducing conditions), we assume that some chemical modification is responsible for its different electrophoretic mobility. The indistinguishability of the protein species by mass spectrometry hints at a rather small mass difference, as may be due to U. Schmucker (Klinikum of the University of Essen) for nucleotide sequencing.

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