

Glyceraldehyde-3-Phosphate Ferredoxin Oxidoreductase from *Methanococcus maripaludis*[▽]

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The genome sequence of the non-sugar-assimilating mesophile *Methanococcus maripaludis* contains three genes encoding enzymes: a nonphosphorylating NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR); all these enzymes are potentially capable of catalyzing glyceraldehyde-3-phosphate (G3P) metabolism. GAPOR, whose homologs have been found mainly in archaea, catalyzes the reduction of ferredoxin coupled with oxidation of G3P. GAPOR has previously been isolated and characterized only from a sugar-assimilating hyperthermophile, *Pyrococcus furiosus* (GAPOR_{pf}), and contains the rare metal tungsten as an irreplaceable cofactor. Active recombinant *M. maripaludis* GAPOR (GAPOR_{mm}) was purified from *Escherichia coli* grown in minimal medium containing 100 μ M sodium molybdate. In contrast, GAPOR_{mm} obtained from cells grown in medium containing tungsten (W) and W and molybdenum (Mo) or in medium without added W and Mo did not display any activity. Activity and transcript analysis of putative G3P-metabolizing enzymes and corresponding genes were performed with *M. maripaludis* cultured under autotrophic conditions in chemically defined medium. The activity of GAPOR_{mm} was constitutive throughout the culture period and exceeded that of GAPDH at all time points. As GAPDH activity was detected in only the gluconeogenic direction and GAPN activity was completely absent, only GAPOR_{mm} catalyzes oxidation of G3P in *M. maripaludis*. Recombinant GAPOR_{mm} is posttranscriptionally regulated as it exhibits pronounced and irreversible substrate inhibition and is completely inhibited by 1 μ M ATP. With support from flux balance analysis, it is concluded that the major physiological role of GAPOR_{mm} in *M. maripaludis* most likely involves only nonoptimal growth conditions.

Glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) catalyzes a dual substrate electron-transfer reaction in which oxidation of glyceraldehyde-3-phosphate (G3P) yields 3-phosphoglycerate (3PG) concomitant with reduction of oxidized ferredoxin. GAPOR and its homologs are found mainly in archaea including both euryarchaeota and crenarchaeota and especially in hyperthermophiles (26, 38, 42). Homologs can also be found in draft sequences of deltaproteobacterial species (M. A. Aklujkar, J. E. Butler, G. Reguera, E. S. Shelobolina, A. L. Lapidus, D. Bruce, and P. M. Richardson, presented at the 106th General Meeting American Society for Microbiology, Orlando, FL, 21 to 25 May 2006). It has been proposed that the role of GAPOR is to replace the conventional two-step reduction of G3P to 3PG catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 3-phosphoglyceric acid phosphokinase (PGK) in only the glycolytic direction of the Embden-Meyerhof-Parnas pathway (26). GAPOR uses ferredoxin as an electron acceptor while GAPDH uses NAD(P)⁺. A glycolytic pathway dependent on GAPOR is supposed to be a primordial pathway in early evolution (38) since at least some ferredoxins are more stable and more thermodynamically preferable for electron transport than pyridine nucleotides at high temperatures (36).

In *Pyrococcus furiosus*, a hyperthermophilic archaea, it has been suggested that the GAPOR reaction is coupled to syn-

thesis of molecular hydrogen as the final electron acceptor (26). Nevertheless, GAPOR is found not only in hydrogen-producing species, such as *Thermococcus celer* as an active enzyme (38) and in *Pyrococcus abyssi* (10) and *Thermococcus kodakarensis* (13) as hypothetical homologs, but also in non-hydrogen-producing, i.e., hydrogen-consuming species, such as *Desulfurococcus amylolyticus* as an active enzyme (38), and *Thermoproteus tenax* (39), *Methanocaldococcus jannaschii* (8), and *Methanococcus maripaludis* (17) as hypothetical homologs. The rationale behind the evolution of a GAPOR-dependent glycolytic pathway in place of a GAPDH-PGK-dependent pathway, particularly in the latter group of organisms, remains unsolved.

M. maripaludis is the sole example so far of a mesophile that contains a gene annotated as GAPOR (GAPOR_{mm}) in its genome sequence (43). *M. maripaludis* is a facultative autotroph (18) which is capable of autotrophic growth in mineral medium in the presence of hydrogen and carbon dioxide. On the other hand, *M. maripaludis* readily incorporates acetate and a variety of amino acids when they are present (44, 45). The amino acids are incorporated into protein but are not subject to further catabolism (45). *M. maripaludis* has also been reported to be capable of synthesis of intracellular glycogen reserves (46). This was suggested from the result that activities of glycolytic enzymes were enhanced while the intracellular glycogen content increased (46). However, *M. maripaludis* has never been reported to be able to assimilate any extracellular sugars. It is therefore surprising that *M. maripaludis* possesses a homolog of *P. furiosus* GAPOR (GAPOR_{pf}) in its genome despite limited opportunities for glycolysis. Furthermore, *M.*

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maripaludis also possesses two homologs of putative NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenases (phosphorylating [GAPDH] and nonphosphorylating [GAPN]), which all in theory are capable of catalyzing the G3P-dependent step of the glycolytic pathway. Yu et al. (46) earlier performed GAPDH assays using crude extracts of *M. maripaludis*; however, no genome sequence was available, and GAPOR had not yet been discovered at that time.

The present study was initiated with an effort toward understanding the physiological role of a gene product which at first instance would not be expected to play an important role in an organism best known for its autotrophic growth capability. Toward this aim, we verified the catalytic function of GAPOR_{Mm} through synthesis and purification of the recombinant protein in *Escherichia coli* and investigated its biochemical and biophysical characteristics with a particular focus on metal specificity. Subsequently, we investigated the relationship between the three enzymes (GAPDH, GAPN, and GAPOR) that are all theoretically capable of catalyzing G3P metabolism in *M. maripaludis*.

MATERIALS AND METHODS

Media and growth conditions of *M. maripaludis*. Techniques used for growing methanogens were those of Miller et al. (25); a serum bottle capped with a butyl rubber inner cap and aluminum sealer was used for cultivation. *M. maripaludis* ATCC 43000, a strain which is not identical with the S2 strain used for genomic sequencing (17), was grown at 37°C in MPI (DSM medium 141 [http://www.dsmz.de/microorganisms/html/media/medium000141.html] with omission of acetate, yeast extract, and Trypticase) which was sparged (H₂-CO₂ [ratio of 80/20], liquid for 5 min; headspace for 30 s) prior to autoclaving. While the medium was hot, 17% cysteine · H₂O · HCl (wt/vol) was added through a 0.2-μm-pore-size filter until the medium lost the color of the redox indicator. The inocula were 10% (vol/vol) of cells in the mid-exponential phase. After the cells were inoculated, the headspace was overpressurized to ~2 atmospheres with the same gas filtered through a 0.2-μm-pore-size filter (Millex; Millipore). For GAPOR assays, cells were harvested on a 0.2-μm-pore-size filter from 5 ml of culture in an N₂-CO₂-H₂ (90/5/5 ratio) atmosphere and lysed by the addition of BugBuster Reagent (Novagen) containing nonionic surfactants under the same atmosphere, with or without the addition of 4% (vol/vol) Triton X-100. The protein concentration in the lysate was determined using a detergent-compatible protein assay (DC Protein Assay; Bio-Rad). The cell lysate was stored at -80°C under the same atmosphere until use. For transcript assays, cells were harvested by centrifugation from 1 ml of culture under the same atmosphere. Three individual cultures were treated the same way.

Transcript analysis. Total RNA was prepared using an EZNA Fungal RNA Miniprep Kit (Omega Bio-Tek, Inc.) according to the manufacturer's instructions. For real-time PCR assays, 30 ng of total RNA was used as a template. Primers GdF (5'-GTAGCTAAACAGGACGATATGAAAG) and GdR (5'-TCAACTACAATATCTGCATCTTCAA) for GAPDH (locus MMP0325), GnF (5'-GAATCAATATCTAAAAATGCGAAAA) and GnR (5'-ATGTTTCAACAACTTCTTCTTCAAC) for GAPN (locus MMP1487), and GpF (5'-GTAGCTAAACAGGACGATATGAAAG) and GpR (5'-TCAACTACAATATCTGCTATCTTCAA) for GAPOR (locus MMP0945) were designed to yield fragments of approximately equal size (200 bp). Primers 16SF (5'-ATGCGAGTCTATGGTTTCGGCCATG) and 16SR (5'-GACGCTTTAGGCCCAATAAAAGTGG) were designed to amplify 16S rRNA as an internal control. Real-time PCR was performed with an AccessQuick reverse transcription-PCR System (Promega). Following the reverse transcription reaction (at 45°C for 45 min), PCR was performed according to the following conditions: 95°C for 2 min and 40 cycles of 95°C for 30 s, 58°C for 30 s, and 68°C for 1 min. The PCR products were analyzed by agarose gel electrophoresis. Reverse transcriptase was excluded from negative controls. Relative expression levels of target genes were quantitated by the comparative cycle threshold method (4) using 16S rRNA as an internal control.

Cloning of GAPOR. Genomic DNA was extracted from actively growing *M. maripaludis* using a DNeasy Tissue Kit (QIAGEN) after repeated cycles of freezing and thawing (3). The entire open reading frame (accession number CAF30501; locus MMP0945) of the gene encoding GAPOR_{Mm} was amplified by PCR using the following oligonucleotide primers: 5'-GACGACGACAAGATG

AACATTTTGATTGATGG and 5'-GAGGAGAAGCCCGTTTATTCTTTT AATTTCAG in the presence of 8% (vol/vol) dimethyl sulfoxide and cloned into pET46 Ek-LIC vector (Novagen) using T4 DNA polymerase (Novagen). As the reverse primer included a stop codon, the pET46-Ek/LIC-GAPOR vector was expected to generate a fusion protein encoding only an N-terminal His tag. The DNA was used to transform *E. coli* strains BL21(DE3) or Rosetta-gami 2(DE3) (Novagen).

Preparation and purification of protein samples. *E. coli* was cultured (37°C; aerobic) in M9 minimal medium (0.8 g/liter of NH₄Cl, 0.5 g/liter of NaCl, 7.5 g/liter of Na₂HPO₄ · 2H₂O, 3.0 g/liter of KH₂PO₄), to which the following separately sterilized components were added (per liter of medium): 2 ml of 1 M MgSO₄ · 7H₂O, 1 ml of 0.1 M CaCl₂, 0.3 ml of 1 mM filter-sterilized thiamine-HCl, and 10 ml of a trace element solution containing (per liter) 1 g of FeCl₃ · 6H₂O, 0.18 g of ZnSO₄ · 7H₂O, 0.12 g of CuCl₂ · 2H₂O, 0.12 g of MnSO₄ · H₂O, and 0.18 g of CoCl₂ · 6H₂O. Sterilized glucose was added to a final concentration of 2 g per liter. Any of the following was added further (per liter of medium): 10 ml of 10 mM Na₂WO₄ · 2H₂O, 10 ml of 10 mM Na₂MoO₄ · 2H₂O, 5 ml of 10 mM Na₂WO₄ · 2H₂O and 5 ml of 10 mM Na₂MoO₄ · 2H₂O, or 10 ml of deionized ultrafiltered water. For M9 medium precultures, the last component was used. A total of 40 mg/liter of L-leucine was added to all media for cultivation of Rosetta-gami 2(DE3) cells. Large-scale expression cultures were conducted using either a 10-liter fermentor (MBF-1000; Eylea, Japan) with constant aeration of 0.1 MPa (at 120 rpm and 37°C) or 1- to 5-liter shake flasks (at 200 rpm and 37°C). Expression was induced at an optical density at 600 nm of 0.5 to 0.6 or of 0.1 for the 1- or 8-liter scale culture, respectively, with isopropyl-β-D-thiogalactopyranoside (IPTG), and bacteria was grown for 12 h. Alternatively, bacteria was grown in the same culture sparged with N₂ (4 ml/min) after the addition of IPTG for anaerobic expression using a Minifor system (Lambda). The bacteria were harvested by centrifugation (8,000 × g for 20 min at 4°C), washed, and resuspended in cold phosphate buffer (10 mM; pH 8.0), and then lysed with BugBuster Reagent (Novagen) containing benzonase nuclease (Novagen) and 1 mM phenylmethylsulfonyl fluoride. Anaerobically induced samples were harvested and lysed under an N₂ or N₂-CO₂-H₂ atmosphere. The lysate was centrifuged (20,000 × g for 20 min at 4°C), and the pellet was washed in buffer A (10 mM phosphate buffer [pH 8.0], 1 mM EDTA, 4% [wt/vol] Triton X-100), centrifuged (20,000 × g for 20 min at 4°C), and resuspended in buffer A containing 8 M urea. The resuspended pellet was dialyzed (for 3 h at 4°C) against 20 mM buffer B (buffer A with the addition of 10 mM urea and 100 mM NaCl) and subsequently, under oxygen-limited conditions, against buffer B with the addition of 1 mM reduced glutathione and 0.2 mM oxidized glutathione (for 3 h at 4°C). Finally, the protein preparation was centrifuged (25,000 × g for 1 h at 4°C), and the supernatant was recovered. Protein preparation using GAPOR_{Mm} expressed in Rosetta-gami 2(DE3) differed from that of BL21(DE3), as the supernatant obtained after the first centrifugation following lysis contained the majority of the activity. In this case, the addition of Triton X-100 and urea was not required for solubilization of GAPOR_{Mm}. GAPOR_{Mm} was purified to >75% homogeneity through a HisTrap HP (GE healthcare) column and gel filtration using Superdex 200 10/300 (GE healthcare) calibrated with low-molecular-weight and high-molecular-weight gel filtration calibration kits (GE healthcare). Protein preparations were routinely monitored with an Agilent 2100 Bioanalyzer.

Cofactor analysis. The purified protein was subjected to metal content analysis using inductively coupled plasma-mass spectrometry ([ICP-MS] SPQ-9000 with SII nanotechnology) after wet-sample digestion by 5 ml of HNO₃ and 1 ml of HClO₄ (11). Controls were conducted using [2Fe-2S]-ferredoxin from spinach (Sigma) and bovine serum albumin (Sigma).

Pterin content was measured using 0.5 mg of GAPOR by the previously described methods (24, 32) with fluorescence detection (lambda excitation at 363 nm; lambda emission at 442 nm). The pterin content was calculated by comparison with pterin-6-carboxylic acid. Xanthine oxidase from buttermilk (Sigma) was used as a positive control.

Acid-labile sulfide was measured by a method described elsewhere (5, 29). The positive control was Na₂S · 9H₂O, and 1 nmol of S²⁻ gave an A₆₇₀ of 0.0014.

Enzyme assay. All assays were conducted in screw-cap cuvettes (Sigma) with butyl rubber caps (Voigt Global Distribution, LLC, Kansas City, KS) at room temperature. All assay mixtures (excluding protein) and headspace were sparged with N₂ (99.9999%) for 2 min and 15 s, respectively; protein was added under a N₂ atmosphere, and thereafter cuvettes were capped. GAPOR activity was routinely measured as previously reported by Mukund et al. (26) by following the reduction of benzyl viologen (BV) at A₅₀₀ (ε_{BV} of 7,400 M⁻¹ cm⁻¹) (1). The reaction mixture contained 30 μM G3P, 3 mM BV, 56 μM Na₂MoO₄, 50 mM EPPS (N-[2-hydroxyethyl]piperazine-N'-[3-propanesulfonic acid]) buffer, pH 8.4. G3P-dependent reduction of ferredoxin was monitored by following the reduction of metronidazole (MNZ) at A₃₂₀ (ε_{MNZ} of 9.3 mM⁻¹ cm⁻¹) at room

temperature, as modified from the procedure of Soboh et al. (40). The reaction mixture contained 30 μ M G3P, 2 mM *Clostridium pasteurianum* ferredoxin, 0.1 mM MNZ, 56 μ M Na₂MoO₄, and 50 mM EPPS buffer, pH 8.4. *C. pasteurianum* ferredoxin was used instead of *M. maripaludis* ferredoxin, which is not available from commercial resources. The assays were started with the addition of 63 mM cysteine-HCl, which is added to ensure completely anoxic conditions, using syringes. Extracts prepared aerobically from aerobically grown cells yielded a reversibly inactivated protein preparation as GAPOR activities measured in anoxic buffer under N₂ atmosphere could be recovered after the addition of L-cysteine. Without the reducing agent, GAPOR reacted very slowly (38-fold decrease with 100 μ M G3P). The presence or absence of oxygen during expression and protein purification did not influence total yield of GAPOR activity or the requirement for L-cysteine-dependent activation.

Cofactor inhibition was tested using 0.1 mM G3P by the addition of either AMP, NADH, NADPH (all at 10 μ M), or ATP (1 and 10 μ M) in the reaction mixture using GAPOR_{Mm} synthesized with Rosetta-gami 2(DE3). To maintain pH above 8.0 in reaction mixtures to which ATP was added, as confirmed using a pH indicator paper (Whatman), the concentration of EPPS buffer in each reaction mixture was increased to a 100 mM final concentration, including control reactions to which no cofactor was added.

Reverse GAPOR activity, i.e., catalysis of G3P formation, was measured by following the oxidation of prereduced BV at A₆₀₀. The reaction mixture contained 100 μ M 3PG, 100 μ M BV, 56 μ M Na₂MoO₄, and 50 mM EPPS buffer, pH 8.4. Assays were started by the addition of 100 μ M sodium dithionite using syringes.

GAPDH activity was determined in both glycolytic and gluconeogenic directions as described previously (47) with minor modifications. The assay mixtures contained 2 mM NADP⁺, 0.3 mM G3P, 50 mM Tris-Cl (pH 8.0), with or without 5 mM K₂HPO₄ (glycolytic direction) or 0.2 mM NADPH, 30 mM 3-phospho-D-glyceroyl phosphate, 30 mM ATP, and 50 mM Tris-Cl (pH 8.0) (gluconeogenic direction). Each assay was started by the addition of protein. The reduction of NADP⁺ and oxidation of NADPH were monitored by fluorescence spectrometry. GAPN activity was determined as for GAPDH activity above, except with the omission of ATP.

Negative control reaction assays were conducted using reaction mixtures in which either protein, G3P, or electron acceptor was omitted. No activity was observed in any control reaction. Omission of IPTG during expression of recombinant GAPOR_{Mm} resulted in complete loss of measurable GAPOR activity in crude lysates. One unit of activity is defined as 1 μ mol of G3P oxidized per min.

Flux balance analysis. Flux balance analysis was performed using the condensed stoichiometric model of *M. maripaludis* and *Desulfovibrio vulgaris* syntrophic coculture developed by Stolyar et al. (41). The input files of the published model were imported without modification and analyzed using the software CellNetAnalyzer (21), Matlab (version 7.0), and the Matlab Optimization module (Mathworks). The following constraints were fixed prior to flux optimization: lactate uptake (D86), 10; ethanol synthesis (D78), 0; glycerol synthesis (D83), 0; SO₄ uptake (D01), 0; alanine uptake (M79), 0. If GAPDH (M32) is constrained to zero prior to flux optimization, all values reported in Fig. 2 of Stolyar et al. (41) are replicated with the exception of reaction M16. In a modified model, changes were incorporated in the reactions catalyzed by GAPDH (M32, 1 ZM_GAP + 1 ZM_ADG = 1 ZM_3PG + 1 ZM_ATP + 1 ZM_NADH; change to 1 ZM_GAP + 1 ZM_ADG = 1 ZM_3PG + 1 ZM_ATP + 1 ZM_NADPH) and GAPOR (M33, 1 ZM_GAP + 1 ZM_ADG = 1 ZM_3PG + 1 ZM_ATP + 1 ZM_Fdx(red); change to 1 ZM_GAP = 1 ZM_3PG + 1 ZM_Fdx(red), an irreversible reaction). To simulate the effect of variation in H₂ availability, reaction M15 (uptake of H₂ by *M. maripaludis*) was constrained in a range between 0 to 16.2 μ mol/h prior to flux optimization.

RESULTS

Amino acid sequence analysis of known and putative GAPOR-encoding genes. The sequence of the gene encoding GAPOR_{Mm} displays 44% similarity at the amino acid level to the gene encoding GAPOR_{Pf}. Ligands to a [4Fe-4S] cluster (e.g., C298, C302, C306, and C514) and molybdopterin (e.g., R60 and D348), which were identified in the GAPOR_{Pf} amino acid sequence (9, 42) and which define other aldehyde oxidoreductase (AOR) family protein sequences (22), are completely conserved in GAPOR_{Mm}.

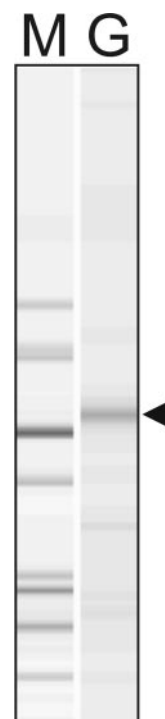


FIG. 1. Electropherogram trace (Agilent 2100 Bioanalyzer) of purified GAPOR (Mo-GAPOR). M, molecular marker (Protein 200 Plus Ladder; Agilent); G, purified Mo-GAPOR (arrowhead indicates the expected size of GAPOR).

The GAPOR activity of cell extracts of *E. coli* expressing GAPOR_{Mm} is dependent upon Mo and inhibited by the addition of W. The gene encoding GAPOR_{Mm} was subcloned into pET46 and first expressed in *E. coli* BL21(DE3). All of the measurable activity was precipitated after centrifugation of the cell extract even though prediction of the solubility of GAPOR_{Mm} (<http://sosui.proteome.bio.tuat.ac.jp>) based on the amino acid sequence suggested that GAPOR_{Mm} is a soluble protein. Attempts to enhance the solubility of GAPOR_{Mm} by expression as a glutathione S-transferase or a maltose binding protein fusion in strain BL21(DE3) resulted in lower yields of total activity and no changes with respect to solubility compared to pET46 (data not shown).

To test the metal requirement of GAPOR_{Mm}, expression was conducted in M9 minimal medium with four variations of metal additions: (i) 100 μ M sodium tungstate (M9/W⁺), (ii) 100 μ M sodium molybdate (M9/Mo⁺), (iii) 50 μ M sodium tungstate–50 μ M sodium molybdate (M9/Mo⁺/W⁺), or (iv) without the addition of either sodium tungstate or sodium molybdate (M9/–). All cultures were induced with 1 mM IPTG at the exponential phase of growth, and the growth rate after induction was comparable in all media (data not shown). Synthesis of a polypeptide of the expected molecular weight of GAPOR_{Mm} was observed in cells grown in all media (Fig. 1). However, measurable GAPOR activity could only be detected in M9/Mo⁺-grown cells (Table 1).

GAPOR_{Mm} catalyzes a BV- and G3P-dependent oxidoreductase reaction with pronounced substrate inhibition. His₆ affinity-purified GAPOR_{Mm} eluted as a single major peak when

TABLE 1. Characteristics of recombinant GAPOR purified from minimal medium containing different combinations of Mo and W

Strain and culture condition	Mass (kDa)	GAPOR yield (mg/g of wet cells)	Specific activity (U/mg of protein) ^a	Amt detected (g-atoms/mol) of indicated substance ^b				
				Mo	W	Fe	Pterin	Acid-labile sulfide
BL21(DE3)								
W	71	5.9	UD	0.013	0.020	ND	ND	ND
Mo	71	4.4	14	0.17	0.0007	1.3	0.34	6.1
W+Mo	71	5.8	UD	0.010	0.022	ND	ND	ND
No Mo/no W	71	3.5	UD	0.012	0.022	ND	ND	ND
Rosetta-gami 2(DE3)								
W	71	0.58	UD	UD	UD	ND	ND	ND
Mo	71	0.65	120	0.74	UD	ND	ND	ND

^a UD, undetectable.^b ND, not determined; UD, undetectable.

subjected to size-exclusion gel chromatography with an estimated molecular mass of 70 kDa. The predicted molecular mass of the amino acid sequence encoding GAPOR_{Mm} is 70.9 kDa (http://tw.expasy.org/tools/pi_tool.html), suggesting that recombinant GAPOR_{Mm} was purified in a monomeric state. No loss of total activity was observed as a result of the His tag purification procedure (data not shown). GAPOR_{Mm} purified from M9/Mo⁺ cultures (Mo-GAPOR) was reversibly inactivated by exposure of liquid preparations to air and reversibly activated by the addition of the reducing agent L-cysteine (data not shown). In contrast, the GAPOR activity of crude lysates of *M. maripaludis* was irreversibly inactivated by exposure to air. Most other previously characterized nonrecombinant AOR proteins (including GAPOR_{Pf}) also appear to be irreversibly inactivated by oxygen exposure (16, 26, 28). The response of recombinant GAPOR_{Mm} to L-cysteine reactivation may be related to the sulfide activation observed for a W-containing formaldehyde oxidoreductase purified from *P. furiosus* (34).

An estimation of kinetic constants for G3P was attempted in which the concentration of G3P was initially varied from 0.01 to 2 mM with the concentration of BV fixed at either 0.5 or 3 mM (Fig. 2). However, the catalytic rate was maximal at 0.1 mM G3P and significantly inhibited above 0.15 mM at both concentrations of BV. GAPOR_{Pf} and AOR of *P. furiosus* also displayed substrate inhibition for each respective aldehyde (28), although less extreme than that of recombinant GAPOR_{Mm}. To probe the nature of the inhibition, Mo-GAPOR was incubated with either 2 mM or 0.1 mM G3P for 10 min. Subsequently, the protein was desalted using Sephadex G-25 and then back-added to assay reaction mixtures containing 0.1 mM G3P and 3 mM BV. Reaction mixtures in which Mo-GAPOR had been preincubated with 2 mM G3P displayed 13-fold lower activity than Mo-GAPOR preincubated with 0.1 mM G3P, suggesting that the inhibition was both irreversible and independent of BV. Substrate inhibition was not observed for BV (Fig. 2B) as previously reported for other AORs. Kinetic constants were not estimated as a result of the severe substrate inhibition for G3P. The physiological electron acceptor of the general GAPOR reaction is proposed to be a ferredoxin, based on kinetic analysis with GAPOR_{Pf} (26) in which a 70-fold greater affinity was observed with *P. furiosus* [4Fe-4S]-ferredoxin than with BV. As a ferredoxin from *M. maripaludis* was

unavailable, a ferredoxin-dependent reaction was instead confirmed using a commercially available [4Fe-4S]-ferredoxin preparation from *C. pasteurianum* with MNZ as a reporter molecule. The reduction of MNZ was dependent upon the

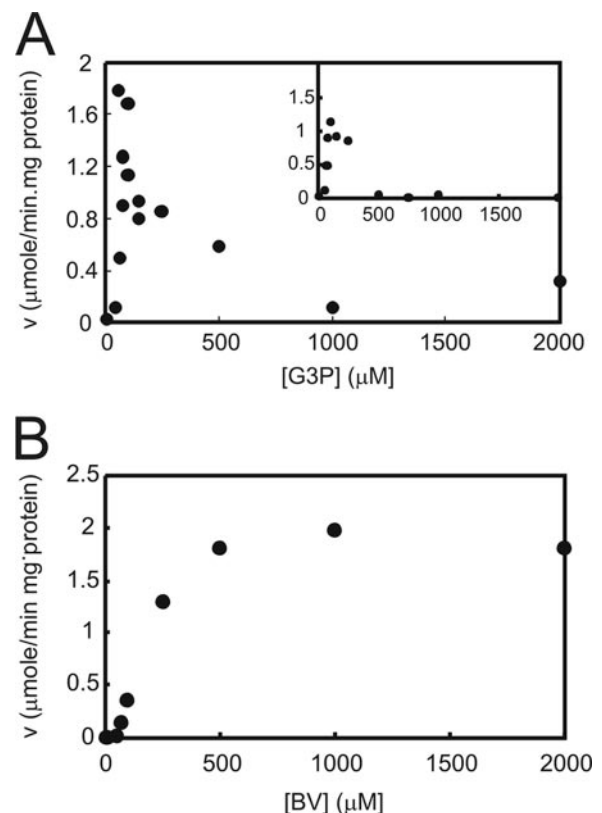


FIG. 2. Effect of variation (v) in concentrations of G3P (A) and BV (B) on the activities (v) of purified Mo-GAPOR synthesized using BL21(DE3). The activities were measured at a BV concentration fixed at 0.5 mM (inset) and 3 mM (A) or a concentration of G3P fixed at 0.1 mM (B). The concentration of the second substrate was varied from 10 to 2,000 μ M. No activity was detected when substrates were omitted. The substrate inhibition pattern was confirmed with Mo-GAPOR synthesized using Rosetta-gami 2(DE3). As the inhibition was nearly identical, only data obtained with GAPOR_{Mm} synthesized with BL21(DE3) are shown.

TABLE 2. Changes in the gene expression profile and enzyme activity of G3P-related enzymes

Day postinoculation	Relative gene expression ratio ^a			Enzyme activity (U/mg of protein) ^b				OD ₆₀₀ ^c
	GAPDH	GAPN	GAPOR	GAPDH (G3P oxidation)	GAPDH (G-1,3-P reduction)	GAPN	GAPOR	
2	UD	UD	0.18	UD	1.9	UD	13	0.057
8	UD	UD	0.0031	UD	2.4	UD	23	0.041
12	0.00011	UD	0.027	UD	0	UD	72	0.065
15	UD	UD	0.019	UD	0.078	UD	25	0.037

^a The relative expression ratio was determined by the comparative cycle threshold method (5) using 16S rRNA as an internal control. UD, over the threshold (i.e., >45 cycles).

^b UD, undetectable.

^c Optical density at 600 nm. This value was 0.003 after inoculation (i.e., day 0).

addition of both Mo-GAPOR and ferredoxin (data not shown).

A maximum specific activity of 1.8 U/mg of protein was observed for Mo-GAPOR expressed in strain BL21(DE3), almost 200-fold lower than that observed with purified GAPOR_{Pf} (26). In order to enhance the activity, alternative host strains were tried for recombinant protein synthesis. Expression of GAPOR_{Mm} in strain Rosetta-gami 2(DE3) in M9 medium with the addition of either Mo or W generated protein which, after purification to apparent homogeneity, yielded specific activities of 120 and 0 U/mg of protein, respectively (Table 1). Using the Rosetta-gami 2(DE3)-derived protein preparation, substrate inhibition was confirmed (data not shown), and the effect of physiological levels of key cofactors was also tested. The addition of a 10 μ M concentration of either AMP, NADH, or NADPH to the enzyme assay reaction mixture had no effect on GAPOR activity although the addition of 1 to 10 μ M ATP resulted in complete loss of GAPOR activity.

In vivo, it is assumed that GAPOR catalyzes only oxidation and not the formation of G3P, as the change in standard free energy of the reverse GAPOR reaction is far greater than the reverse PGK-GAPDH reaction due to a lack of ATP hydrolysis in the former case. As comparative estimates of actual free-energy changes in vivo are not easily calculated, particularly due to a lack of information regarding the relative ratio of reduced/oxidized ferredoxin in vivo, we instead tested whether Mo-GAPOR is enzymatically capable of catalyzing the reverse reaction in vitro. However, Mo-GAPOR was unable to catalyze 3PG-dependent oxidation of reduced BV (data not shown).

Metal and cofactor analysis of GAPOR_{Mm}: the Mo-content of GAPOR_{Mm} correlates with catalytic activity. The metal content of acid hydrolysates of purified GAPOR_{Mm} preparations was analyzed by ICP-MS (Table 1). Negative controls for ICP-MS using an equal amount (compared to GAPOR_{Mm}) of bovine serum albumin or spinach [2Fe-2S]-ferredoxin did not contain detectable amounts of W and Mo (detection limit of <2.3 ppt for W and <0.3 ppt for Mo). The only catalytically active preparation (Mo-GAPOR) contained 10-fold greater levels of Mo than that derived from any other combination of metal additions and more than 100 times the amount of Mo than W.

Molybdopterin extracted from Mo-GAPOR emitted fluorescence with a peak at 442 nm when excited with light filtered at 363 nm with a bandwidth of 5 nm. The molybdopterin content of Mo-GAPOR was compared with a positive control consist-

ing of pterin-6-carboxylic acid. The molybdopterin content of Mo-GAPOR was estimated to be 0.35 g-atoms per mol of protein, approximately twice the molar content of Mo. Mo-GAPOR was estimated to contain 6.1 g-atoms of acid-labile sulfide per mol of protein. Although the content of acid-labile sulfide was more than that of iron, disagreements in the relative content of Fe and S²⁻ have been observed in other Fe-S-containing proteins (6, 19, 23).

Based on analysis of *P. furiosus* AOR and GAPOR_{Pf} by Mukund and Adams (26, 28) and a comparison between the sequences coding for GAPOR_{Mm} and GAPOR_{Pf}, we expect ca. 1 g-atoms of W or Mo/mol and ca. 5 g-atoms of Fe/mol and S²⁻/mol if all recombinant GAPOR_{Mm} molecules are fully intact and assembled correctly. Hence, both the ICP-MS and pterin analysis suggest that only one-fifth of Mo-GAPOR had acquired a molybdopterin cofactor and iron sulfur cluster.

Purified GAPOR_{Mm} that had been expressed using Rosetta-gami 2(DE3) instead of BL21(DE3) in M9 medium with the addition of Mo contained 0.74 g-atoms of Mo and undetectable amounts of W (Table 1). No trace of the two metals was detected in GAPOR_{Mm} expressed in Rosetta-gami 2(DE3) using M9 medium with the addition of W only. As the difference in specific activity between GAPOR_{Mm} derived from BL21(DE3) and Rosetta-gami 2(DE3) was greater than the relative difference in Mo content, this suggests that the change in host affected more than just the Mo content of GAPOR_{Mm}.

GAPOR catalyzes G3P oxidation and GAPDH catalyzes G3P synthesis in *M. maripaludis* grown under autotrophic conditions. *M. maripaludis* was grown under autotrophic growth conditions in chemically defined medium containing added Mo and no added W (growth under these conditions is dependent on the presence of Mo). RNA and protein were extracted at several time points and analyzed for enzyme activity and transcript levels. As the yield of RNA was not sufficient to allow for Northern analysis (data not shown), real-time PCR was conducted instead.

GAPOR activity reached a maximum at day 12 and then decreased (Table 2). GAPDH activity was observed in the gluconeogenic direction only, and the activity was higher in early cultures and decreased thereafter. GAPOR-encoding transcript was observed at all time points, while only one cDNA preparation yielded detectable GAPDH transcript. No activity and no transcript encoding GAPN were detected at any stage of the culture period. Although there were discrepancies between the presence or absence of transcript and total enzyme activities, both data sets suggest that GAPOR is the

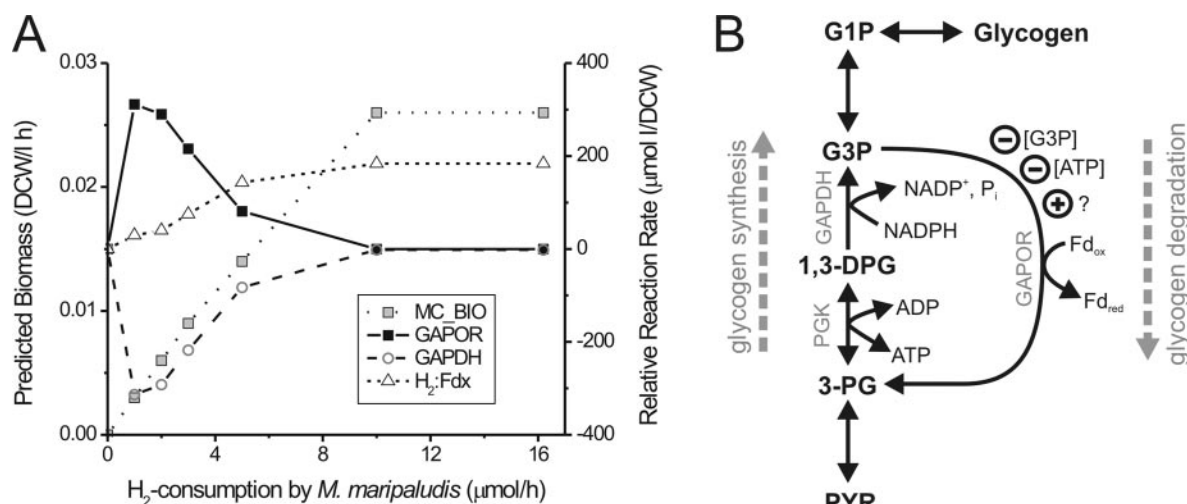


FIG. 3. (A) The predicted effect of H₂ availability on biomass yield (MC BIO) and relative reaction rate of H₂-dependent reduction of ferredoxin (H₂:Fdx), GAPDH in the direction of G3P-oxidation, and GAPOR in the direction of G3P-oxidation. For each fixed H₂ value (constrained prior to flux optimization), the predicted reaction rate of each reaction was divided by the predicted biomass yield (left y axis) to obtain the reaction rate relative to biomass yield (right y axis). (B) Graphic summary of proposed pathways and regulation of glycogen metabolism in *M. maripaludis*. Only reactions related to the present study are shown; other reactions (amino acid metabolism and pentose phosphate pathway) utilizing the same metabolites have been omitted. Regulating molecules are indicated by a circle with a minus sign and, in the case of an unknown putative positive effector, by a plus sign. Abbreviations: G1P, glucose-1-phosphate; 1,3-DPG, 1,3-diphosphateglycerate; 3-PG, 3-phosphoglycerate; PYR, pyruvate. DCW, dry cell weight.

dominant G3P-metabolizing enzyme and the only enzyme catalyzing oxidation of G3P under the tested conditions.

Flux balance modeling predicts a zero GAPOR reaction rate under steady-state conditions. Recently, a condensed in silico metabolic model of *M. maripaludis* in syntrophic coculture with *D. vulgaris* was published by Stolyar et al. (41). The published model was based on GAPDH and GAPOR reactions which differed (as summarized in Materials and Methods) from the reactions which in the present study had been verified using crude extracts and purified enzymes. If the GAPDH reaction was not constrained to zero, the original model predicted cyclic flux between GAP and 3PG at a rate close to 5 μmol/h that effectively would yield a net NADH:ferredoxin-oxidoreductase reaction without loss or gain of ATP and GAP. When the two GAPDH and GAPOR reactions were stoichiometrically corrected by changing the nucleotide pyridine cofactor specificity of GAPDH from NAD(H) to NADP(H) and by removing ADP/ATP from the GAPOR reaction, it was no longer necessary to eliminate the GAPDH reaction in order to achieve the published flux values (41). In the corrected model, flux through the irreversible reaction catalyzed by GAPOR was predicted to be zero, while predicted flux catalyzed by GAPDH (−0.6 μmol/h) was substantially reduced. Simulation of GAPOR deletion prior to flux optimization consequently resulted in no change. The effect of variation in H₂ availability was tested by constraining *M. maripaludis* H₂ uptake (reaction M15) between the value obtained under nonlimiting conditions (16.2 μmol/h) and zero (Fig. 3A). Such a condition may be found, for example, if other H₂-consuming microorganisms also are present. When the available H₂ content was reduced, initially the H₂-dependent reduction of F420 was predicted to rapidly become zero (data not shown), and subsequently the H₂-dependent reduction of ferredoxin also decreased (Fig. 3A). At this stage (between 5 to 10 μmol of H₂/h), a cyclic GAPOR-

GAPDH reaction catalyzing an ATP-dependent NADPH:ferredoxin-oxidoreductase reaction was predicted to commence, eventually (i.e., when H₂ uptake was <5 μmol/h) reducing ferredoxin at a greater rate than the hydrogenase-catalyzed reaction.

DISCUSSION

GAPOR_{pf} contains W (26), and it appears specific for W because even in medium containing excess Mo or vanadium (V) and trace amounts of W, no GAPOR_{pf} activity is detected (27). The factors which affect the choice of metals used by enzymes containing molybdenum cofactor (Mo-Co), a cofactor consisting of (i) organic molybdopterin and (ii) inorganic molybdenum or tungsten, remains a question of great interest (20). Kletzin and Adams suggested that W is obligatory in enzyme reactions which require very low redox potentials (≤−420 mV), such as the ferredoxin-dependent oxidoreductases AOR, GAPOR, and formaldehyde ferredoxin oxidoreductase (22). Analysis of recombinant GAPOR_{Mm} expressed in minimal medium clearly indicated that GAPOR containing Mo and pterin is catalytically active and that the reaction catalyzed by GAPOR is not dependent on W. The accumulation of active GAPOR_{Mm} in *M. maripaludis* cultured in chemically defined medium containing Mo but not W further supports the idea that GAPOR_{Mm} containing Mo and not W is catalytically active. This verifies that GAPOR_{Mm} encodes an enzyme with said activity and that the enzyme is actively synthesized under autotrophic growth conditions. The synthesis of active GAPOR containing only Mo suggests that the lack of GAPOR activity in extracts of *P. furiosus* cultured in the absence of W and presence of Mo or V (27) does not result from a chemical incompatibility related to the redox potential of the substrates and end products (22).

The addition of tungstate to the medium did not result in the formation of active Mo-Co containing recombinant GAPOR_{Mm}, even in the absence of added Mo. This is unlikely to be due to a lack of capability by *E. coli* to synthesize W-containing Mo-Co. It has previously been demonstrated that *E. coli* can both incorporate tungstate using the same mechanism for molybdate uptake (33) and synthesize Mo-Co containing both W and Mo (7, 33). In addition, Mo-Co biosynthesis is an ancient, ubiquitous, and highly conserved pathway (31), and *E. coli* is no exception (35). Therefore, that active GAPOR_{Mm} containing a tungsten cofactor was not synthesized is most likely explained, at least in this case, by the metal cofactor specificity of GAPOR_{Mm}. An incompatibility between *E. coli* and GAPOR_{Mm} with respect to maturation factor requirements or the presence or absence of a nucleotide, or a type of nucleotide, would be expected to influence equally the formation of both molybdenum- and tungsten-containing Mo-Co.

Mo-GAPOR is the first Mo-containing AOR member for which the corresponding gene sequence has been verified. Since the [4Fe-4S] cluster and molybdopterin ligands identified in sequences encoding other W-containing AORs (9) are conserved, the root of metal specificity must be determined by other as yet unidentified residues, if not dictated by the organism and/or environments as a whole.

The simultaneous addition of an equal concentration of Mo and W resulted in completely inactive MoW-GAPOR, suggesting that the presence of equimolar amounts of W inhibits the formation of Mo-containing Mo-Co, at least in the special case of GAPOR_{Mm}. However, this should not pose limitations on the synthesis of Mo-dependent enzymes in natural environments since Mo is far more abundant than W in most geographical locations (22). GAPOR_{Mm} therefore most likely evolved to utilize Mo in accordance with the metal abundance of the habitat of *M. maripaludis*, thereby differentiating from "ancestral" hyperthermophilic GAPOR at least with respect to metal specificity.

It has been suggested that GAPOR operates solely in the glycolytic direction (26) while GAPDH and PGK support a gluconeogenic pathway (26, 37) in sugar-utilizing *P. furiosus*. Mukund and Adams (26) also suggested that *P. furiosus* produces 2 mol less ATP per mol of glucose and disposes of excess electrons as molecular hydrogen as a result of exploiting GAPOR instead of GAPDH and PGK (26). This apparently inefficient mechanism may represent an ancestral form of glycolysis at the early stages of the evolutionary process of thermophilic organisms and can be explained by the fact that certain ferredoxins may be more stable than NAD(P)H at high temperatures (38). However, it is puzzling why GAPOR, which catalyzes the reaction in only the glycolytic direction, is found in a facultative autotroph that is not known to assimilate sugars. Furthermore, as the genome of *M. maripaludis* also contains two more candidates potentially capable of catalyzing G3P metabolism (GAPDH and GAPN), it is difficult to understand why ATP generation would be sacrificed if the organism is genetically capable of replacing the GAPOR reaction with ATP-generating GAPDH. We therefore performed a time course study to verify the presence of each of the three genes and encoded enzymes and to verify the directionality of the reactions that they catalyze (Table 2). Transcript and enzyme assays showed that only GAPOR_{Mm} operates in the

glycolytic direction, while only GAPDH operates in the gluconeogenic direction under autotrophic conditions, as the reactions catalyzed by GAPOR_{Mm} and GAPDH were unidirectional with opposing directionality. This contrasts to earlier results (46) in which phosphorylating NADP⁺-dependent G3P oxidation was reported using crude extracts of *M. maripaludis* cultured under conditions similar to those used in the present study. Notably, the glycolytic GAPDH activity reported earlier was >90 and >2,000 times lower than the gluconeogenic GAPDH and glycolytic GAPOR activities, respectively, that were observed in the present study. While catalytically irreversible GAPDH isoenzymes that oxidize G3P (14) or catalytically reversible GAPDH isoenzymes with proposed physiological unidirectional roles (12) have previously been characterized, to our knowledge there are no previous reports of any phosphorylating GAPDH which catalyzes only formation, but not oxidation, of G3P. Further study is required to elucidate the cause of such apparent unidirectionality.

No sign of GAPN-encoding transcript or GAPN activity was observed at any time point of the time course study. It was recently demonstrated that a gene annotated as GAPN in *Methanocaldococcus jannaschii* that shares 56% amino acid identity with *M. maripaludis* GAPN (locus tag MMP1487) in fact encodes an aldehyde dehydrogenase with no specificity for G3P (15). The three residues proposed to be essential for binding of the phosphate moiety of G3P are also missing in the *M. maripaludis* GAPN sequence. Even if the *M. maripaludis* gene annotated as GAPN were expressed, it is likely that the encoded protein in any case would not catalyze nonphosphorylating NAD(P)⁺-dependent G3P oxidation.

Flux balance analysis, using a model corrected with respect to the stoichiometry and directionality of the reactions catalyzed by GAPOR and GAPDH, predicted that glycolytic flux would be strictly limited, at least under steady-state conditions of autotrophic growth, in order to achieve theoretically optimum biomass formation. As only GAPOR_{Mm} is capable of catalyzing G3P catabolism and assuming that no other pathways of glycogen catabolism exist in *M. maripaludis*, this suggests that glycogen catabolism may also be limited under non-steady-state autotrophic growth conditions. Furthermore, although the GAPDH-GAPOR pair, together with other enzymes catalyzing reversible or a combination of irreversible steps of glycolysis/gluconeogenesis, would allow a cycle of glycogen storage and turnover as previously suggested (46), such a cycle of glycogen turnover, or a cycle between pyruvate and G3P alone, that is dependent upon both GAPDH and GAPOR in concert would constitute, in effect, an ATP-dependent NADPH:ferredoxin-oxidoreductase reaction. This raises the questions of how such a reaction cycle and/or glycogen turnover would be regulated and what would be the role of the metabolic reactions catalyzed by GAPDH-GAPOR in *M. maripaludis*.

Both the transcript and enzyme analysis suggested that GAPOR was constitutive throughout the growth period, while GAPDH activity was observed mainly in the early growth stages of the cultures. This contrasts with previous results with *P. furiosus* in which GAPOR_{Pf} was suggested to be transcriptionally controlled and induced by the presence of extracellular sugar (41). As methanogens in general do not assimilate extracellular sugar, a similar regulation mechanism is not possi-

ble with *M. maripaludis*, which may explain why GAPOR activity was constitutive. Regulation may instead be post-transcriptional. Both GAPOR_{Pf} (23) and GAPOR_{Mm} (Fig. 2) are strongly inhibited by G3P; however, no other apparent allosteric interactions were previously noted with GAPOR_{Pf} (23). It is difficult to imagine how the apparently irreversible substrate inhibition exhibited by GAPOR_{Mm} alone would be able to exert any control over glycolytic flux. We therefore tested whether any cofactors would be able to affect the GAPOR-catalyzed reaction. Interestingly, ATP completely inhibited the reaction even at concentrations as low as 1 μ M. As this concentration is lower than intracellular levels typically reported in other microorganisms (30), it is likely that an additional, as yet unknown, positive effector also influences GAPOR activity in vivo. Nevertheless, it is clear that, in contrast to GAPOR_{Pf}, GAPOR_{Mm} is posttranscriptionally regulated.

The results of flux balance analysis and assays determining the molecular regulation of GAPOR_{Mm} and enzyme directionality of GAPOR and GAPDH together allow us to formulate a tentative hypothesis regarding the metabolic role of GAPOR_{Mm}, which is summarized in Fig. 3B. Given that only GAPOR_{Mm} is able to catalyze G3P catabolism and that the enzyme is subject to posttranscriptional regulation, it would appear that GAPOR_{Mm} plays a central role in regulation of glycogen catabolism. The suggested roles of glycogen metabolism in microorganisms vary depending on the species, including temporary conditions of stress imposed by limitations in growth substrate (i.e., starvation) or chemical warfare or undefined continuous cycling (2). As flux balance analysis indicated no role for GAPOR_{Mm} under steady-state autotrophic growth conditions, arguing against a role in continuous cycling, GAPOR_{Mm} and glycogen catabolism are therefore more likely to play a metabolic role under non-steady-state growth conditions (for example, stationary phase or starvation) or under growth conditions dependent on alternative substrates. The fact that ATP is a potent negative regulator of GAPOR_{Mm} further supports the notion that glycogen degradation in *M. maripaludis* may be limited to conditions of starvation, as the concentration of ATP would be expected to be lower under such conditions. Alternatively, the role of ATP as a negative regulator of GAPOR_{Mm} may also be important in order to limit continuous ATP-driven and NADPH-dependent reduction of ferredoxin, a reaction that should be necessary only in the special case where both glycogen and H₂, the major source of electrons for reduction of ferredoxin (41), are limited. In order to test this, we simulated the effect of reduced H₂ availability on predicted reaction rates using flux balance analysis (Fig. 3A). At least stoichiometrically, a cyclic GAPOR-GAPDH reaction was predicted to maximize biomass formation in the event that the uptake of only H₂ and no other external metabolites is limited. Whether such a putative reaction cycle takes place in vivo or not will require further investigation. Nevertheless, it can be concluded that both the proposed role of GAPOR and GAPDH in glycogen catabolism, as well as a putative ATP- and NADPH-dependent reduction of ferredoxin, most likely are important only for maintaining metabolic homeostasis under nonoptimal growth conditions.

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