The α -Glucan Phosphorylase MalP of *Corynebacterium glutamicum* Is Subject to Transcriptional Regulation and Competitive Inhibition by ADP-Glucose

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ABSTRACT

α -Glucan phosphorylases contribute to degradation of glycogen and maltodextrins formed in the course of maltose metabolism in bacteria. Accordingly, bacterial α -glucan phosphorylases are classified as either glycogen or maltodextrin phosphorylase, GlgP or MalP, respectively. GlgP and MalP enzymes follow the same catalytic mechanism, and thus their substrate spectra overlap; however, they differ in their regulation: GlgP genes are constitutively expressed and the enzymes are controlled on the activity level, whereas expression of MalP genes are transcriptionally controlled in response to the carbon source used for cultivation. We characterize here the modes of control of the α -glucan phosphorylase MalP of the Gram-positive *Corynebacterium glutamicum*. In accordance to the proposed function of the *malP* gene product as MalP, we found transcription of *malP* to be regulated in response to the carbon source. Moreover, *malP* transcription is shown to depend on the growth phase and to occur independently of the cell glycogen content. Surprisingly, we also found MalP activity to be tightly regulated competitively by the presence of ADP-glucose, an intermediate of glycogen synthesis. Since the latter is considered a typical feature of GlgPs, we propose that *C. glutamicum* MalP acts as both maltodextrin and glycogen phosphorylase and, based on these findings, we question the current system for classification of bacterial α -glucan phosphorylases.

IMPORTANCE

Bacterial α -glucan phosphorylases have been classified conferring to their purpose as either glycogen or maltodextrin phosphorylases. We found transcription of *malP* in *C. glutamicum* to be regulated in response to the carbon source, which is recognized as typical for maltodextrin phosphorylases. Surprisingly, we also found MalP activity to be tightly regulated competitively by the presence of ADP-glucose, an intermediate of glycogen synthesis. The latter is considered a typical feature of GlgPs. These findings, taken together, suggest that *C. glutamicum* MalP is the first α -glucan phosphorylase that does not fit into the current system for classification of bacterial α -glucan phosphorylases and exemplifies the complex mechanisms underlying the control of glycogen content and maltose metabolism in this model organism.

The α -glucan phosphorylases (EC 2.4.1.1) catalyze the reversible cleavage of α -1,4-glycosidic linkages in polysaccharides, thereby liberating α -glucose-1-phosphate. By this means, α -glucan phosphorylases participate in metabolic processes such as degradation of the intracellular storage polysaccharides glycogen and starch (1, 2), as well as the degradation of maltodextrins formed both in the course of the maltose metabolism of various bacteria (3, 4) and in the cytosol of plant leaves (5–7). Several α -glucan phosphorylases have been extensively studied, their crystal structures have been solved, and the catalytic mechanisms have been described (8–11). Although the catalytic mechanisms appear to be similar in all hitherto characterized phosphorylases (12–14), the enzymes differ dramatically in their substrate preferences, their physiological functions and also, accordingly, their modes of regulation (15). Among the best-known bacterial α -glucan phosphorylases are the two isoenzymes maltodextrin phosphorylase (MalP) and glycogen phosphorylase (GlgP) from *Escherichia coli* (9, 16–19). *E. coli* MalP shows a clear substrate preference for maltodextrins, while the activity with storage carbohydrates containing additional α -1,6-glycosidic linkages such as glycogen and starch is rather low (15, 20). Maltodextrins are formed in *E. coli* by the amylomaltase MalQ as intermediates in the course of maltose utilization (4). The *malQ* and *malP* genes of

E. coli form an operon, whose expression is induced by the transcriptional activator MalT in response to the uptake of maltose (21–23). In addition, transcription of *malPQ* is induced endogenously by MalT in the course of glycogen degradation (24), since the gene products MalQ and MalP also degrade maltodextrins

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formed in the course of glycogen degradation (25). GlgP and the glycogen debranching enzyme GlgX catalyze the initial steps of glycogen degradation in *E. coli* (17, 26), which lead to the formation of maltodextrins (25, 27). The *glgP* gene is localized within the *glgCAP* operon (28, 29) among the genes for the two initial enzymes for glycogen synthesis ADP-glucose pyrophosphorylase (GlgC) and glycogen synthase (GlgA). Thus, in *E. coli* transcriptional control is not applicable for coordination of glycogen synthesis and degradation. Indeed, *E. coli* GlgP is allosterically activated and inhibited by AMP and ADP-glucose, respectively, and additionally allosterically regulated by binding to the phosphocarrier protein Hpr of the phosphoenolpyruvate:sugar phosphotransferase system (18).

The Gram-positive soil bacterium *Corynebacterium glutamicum* is well known for the industrial production of amino acids such as L-glutamate and L-lysine (30) and serves as nonpathogenic model organism to depict metabolism and cell wall biogenesis in closely related mycobacteria (31–33). *C. glutamicum* transiently accumulates large amounts of glycogen in the course of cultivation on sugar substrates (34, 35) and uses maltose efficiently as a substrate for growth and amino acid production (36). The proposed pathways for glycogen metabolism, as well as maltose utilization, in *C. glutamicum* require the action of α -glucan phosphorylases. Indeed, two genes for α -glucan phosphorylases reside in the *C. glutamicum* genome, namely, *glgP1* (*cg1479*) and *glgP2* (*cg2289*) (37). The metabolic pathway for maltose metabolism in *C. glutamicum* is similar to the pathway known from *E. coli* and involves maltose uptake by the ABC transporter MusEFGK₂I, maltodextrin formation by MalQ, degradation of maltodextrins to glucose-1-phosphate (glc-1-P) by an α -glucan phosphorylase, and glucose-6-phosphate (glc-6-P) formation by glucokinases and phosphoglucomutases (38–41). Based on the absence of maltodextrin phosphorylase activity in the *glgP1* deletion mutant strain and the severe growth phenotype of this strain observed exclusively in cultivations on maltose, the *glgP1* gene product was assigned the function of a maltodextrin phosphorylase and the gene therefore was renamed *malP* (41). Since residual α -glucan phosphorylase activity with glycogen as the substrate was detected in extracts of the *glgP1* deletion mutant strain, the *glgP2* gene product was assigned the function of a glycogen phosphorylase (41).

Beside its role in maltose metabolism, MalP also participates in the *C. glutamicum* glycogen metabolism. In cultivations of *C. glutamicum* on glucose, large amounts of glycogen are synthesized in the early exponential growth phase from glc-6-P by the consecutive action of phosphoglucomutase (Pgm), ADP-glucose pyrophosphorylase (GlgC), glycogen synthase (GlgA), and glycogen branching enzyme (GlgB) (34, 40, 42, 43). Degradation of glycogen takes place in *C. glutamicum* before the entry of the stationary phase and proceeds by the concerted action of the glycogen debranching enzyme (GlgX) and the two α -glucan phosphorylases designated MalP and GlgP (41, 43).

The presence of mechanisms for the control of maltose metabolism, as well as for the coordination of glycogen synthesis and degradation, is expected for *C. glutamicum*; however, only the transcriptional control of genes for the maltose uptake system and of the genes *glgC* and *glgA* required for the glycogen synthesis have been investigated thus far (38, 44). Mechanisms for the control of enzyme activities involved in either glycogen degradation or maltose metabolism have not yet been analyzed in *C. glutamicum*. We

investigate here the transcriptional control of *malP*, as well as activity control of the purified MalP protein. In previous RNA microarray studies of the *C. glutamicum* transcriptome, a strong repression of *malP* has been observed for cells cultivated on acetate compared to cells cultivated on glucose (45). Since decreased expressions of both *glgC* and *glgA* lead to a reduced glycogen content in cells cultivated on acetate (44), the reduced expression of *malP* might be caused as a direct response to the culture conditions and/or by the lack of endogenous induction. Therefore, we investigate in this communication also the effects of the deletion of *glgA* and *glgC* on *malP* transcription and MalP activity. The well-characterized α -glucan phosphorylase CcStP from *Corynebacterium callunae* was described as a starch phosphorylase based on its substrate preference for the branched glucose-polymer starch over linear maltodextrins (13, 46–52). Based on the close relatedness of *C. glutamicum* MalP to CcStP, which probably acts as the glycogen phosphorylase in *C. callunae*, we also analyzed the substrate spectrum of *C. glutamicum* MalP as well as control of MalP activity by ATP, ADP, AMP, and ADP-glucose. We show here that, in addition to substrate-dependent transcriptional control of *malP*, the encoded α -glucan phosphorylase MalP is efficiently controlled by competitive inhibition by the glycogen synthesis intermediate ADP-glucose. Based on the characteristics of *C. glutamicum* MalP control, we question the current system for the classification of bacterial α -glucan phosphorylases and discuss the consequences of our findings on the regulation of MalP for the control of glycogen content in *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. *E. coli* and all precultures of *C. glutamicum* were grown aerobically in TY complex medium (53) at 37 and 30°C, respectively, as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. For the main cultures of *C. glutamicum*, cells of an overnight preculture were washed twice with 0.9% (wt/vol) NaCl and then inoculated into CGC minimal medium (54) containing maltose, glucose, or acetate as carbon sources as indicated in Results. When appropriate, kanamycin (25 or 50 μ g ml⁻¹), chloramphenicol (30 μ g ml⁻¹), carbenicillin (30 μ g ml⁻¹), and/or isopropyl- β -D-thiogalactopyranoside (IPTG; 250 μ M) were added to the media. The growth of *E. coli* and of *C. glutamicum* was monitored by measuring the optical density at 600 nm (OD₆₀₀). For analyses of enzyme activities and RNA levels in the course of cultivations, growth experiments were performed aerobically at 30°C as 1-liter cultures in 1.5-liter jars in a Biostat B fermentation system from Braun, essentially as described previously (35). The pH was maintained at 7.0 by online measurement by using a standard pH electrode (Mettler Toledo) and the addition of 4 M KOH and 15% (vol/vol) H₂SO₄. Dissolved oxygen was measured online by use of a polarimetric oxygen electrode (Mettler Toledo), and it was adjusted to 30% of saturation in a cascade by stirring at 100 to 1,200 rpm and aeration with 3 liters of air per min. For enzymatic analysis of intracellular polysaccharides, 5-ml samples of the respective cultures were harvested, cell extracts were prepared, and the glycogen content was determined by using amyloglucosidase as described previously (35). Substrate concentrations in the culture supernatants (maltose and glucose) were analyzed by high-pressure liquid chromatography as described previously (41). The dry weight of *C. glutamicum* in the course of cultivations for the determination of the glycogen content was calculated according to the OD₆₀₀ (as described in the supplemental material, an OD₆₀₀ of 1 corresponded to 0.35 g liter⁻¹ for *C. glutamicum* strains irrespective of the glycogen content; see Fig. S7 in the supplemental material).

DNA isolation, transfer, and manipulations. Standard procedures were used for plasmid isolation, cloning, and transformation of *E. coli*

TABLE 1 Strains used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	F [−] ϕ 80lacZ Δ M15 Δ (lacZYA-argF)U169 phoA supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1	84
BL21(DE3)	ompT hsdS _B (r _B [−] m _B [−]) gal dcm (DE3)	85
<i>C. glutamicum</i>		
WT	Wild-type strain ATCC 13032	American Type Culture Collection
Δ malP mutant	WT with deletion of malP (cg1479)	41
Δ glgAC mutant	WT with deletion of glgA and glgC (cg1268 and cg1269)	This study
IMglgC	WT with insertion of pDrive in glgC (cg1269)	35
Plasmids		
pET16b	Amp ^r pBR322 oriV _{E. coli} P _{T7} , lacI; vector for overproduction of proteins with an N-terminal decahistidine tag in <i>E. coli</i>	Novagen
pET16b-malP	pET16b containing the malP gene	This study
pDrive	Km ^r Amp ^r lacZ α ori1 ori-pUC; PCR cloning vector	Qiagen, Hilden, Germany
pDrive-RACE-PRmalP	pDrive derivative containing the PCR-amplified malP fragment from the RACE assay	This study
pK19mobsacB	Km ^r ; mobilizable <i>E. coli</i> vector for the construction of insertion and deletion mutations in <i>C. glutamicum</i> (oriV sacB lacZ α)	86
pK19mobsacB Δ glgAC	Km ^r ; pK19mobsacB with the deletion construct for the region from cg1268 to cg1269	This study
pET2	Promoter probe vector carrying the promoterless cat gene; Km ^r	87
pET2-PRmalP	pET2 containing the musF promoter fragment	This study
pXMJ19	Expression vector; ptac lacI ^q Cm ^r	88
pXMJ19-malP	pXMJ19 carrying the malP gene	This study

^a Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Km^r, kanamycin resistance.

DH5 α , as well as for electrophoresis (53). *C. glutamicum* chromosomal DNA was isolated as described previously (55). Transformation of *C. glutamicum* was performed by electroporation using the methods of Tauch et al. (56), the recombinant strains were selected on LB-BHIS agar plates containing kanamycin (25 μ g ml^{−1}) and/or chloramphenicol (30 μ g ml^{−1}). Electroporation of *E. coli* was performed according to the method of Dower et al. (57). All enzymes were obtained from New England BioLabs and used according to the instructions of the manufacturer. PCR experiments were performed in a Tprofessional thermocycler (Biometa). Deoxynucleoside triphosphates were obtained from Bio-Budget, and oligonucleotides (primers) were obtained from Eurofins MWG Operon. Cycling times and temperatures were chosen according to fragment length and primer constitution. PCR products were separated on agarose gels and purified using a NucleoSpin extract II kit (Macherey & Nagel). All cloned fragments were verified by sequencing (GATC Biotech AG).

Construction of a reporter gene fusion with the promoter of malP. The promoter probe vector pET2 was used to construct a transcriptional fusion of the malP promoter to the promoterless cat gene. The malP promoter region (from positions −245 to +96 in relation to the malP ATG start codon) was amplified by PCR from genomic DNA of *C. glutamicum* WT by using the primers PRmalP_CAT_for and PRmalP_CAT_rev (see Table S1 in the supplemental material). The 360-bp PCR product was digested with PstI and BamHI and cloned into the vector pET2 cut with the same enzymes, resulting in the plasmid pET2-PRmalP.

Construction of a *C. glutamicum* glgAC deletion mutant. In-frame deletion of the genomic locus comprising glgA and glgC using pK19mobsacB was constructed as described previously (58). In detail, the glgA-glgC gene cluster, including flanking regions, was amplified by using the primer pair DelglgAC_for and DelglgAC_rev (see Table S1 in the supplemental material). The resulting 3,669-bp PCR product was digested with EcoRI and BamHI and cloned into pK19mobsacB cut with the same enzymes. The resulting plasmid was digested with AscI and MluI to remove an internal 1,457-bp fragment of the glgAC region, and the resulting 6,934-bp linear fragment was purified by gel extraction and religated, which then yielded the plasmid pK19mobsacB Δ glgAC. Plas-

mid pK19mobsacB Δ glgAC carries the flanking regions of the glgAC region and was used for gene deletions as described previously (58). The deletion of the glgAC internal region was verified by PCR using the primer pair DglgACContr_for and DglgACContr_rev, resulting in a 3,801-bp fragment for *C. glutamicum* wild-type (WT) and a 2,344-bp fragment for the deletion mutant *C. glutamicum* Δ glgAC.

Overproduction and purification of MalP-HIS. Vector pET16b was used for the synthesis of the N-terminal histidyl (His)-tagged MalP fusion protein. The malP gene was amplified from chromosomal DNA of *C. glutamicum* WT by PCR with primers malPRegion_for and malPRegion_rev using the Q5 DNA polymerase (New England BioLabs). The 2819-bp PCR product was used as the template for a nested PCR with the primers malPHIS_for and malPHIS_rev. The resulting 2,400-bp PCR product was cloned using the primer-added AseI and SalI restriction sites in the NdeI- and XhoI-cut plasmid pET16b. The resulting plasmid, pET16b-malP, was transformed into *E. coli* BL21(DE3). The nucleotide sequence of the malP fragment in plasmid pET16b-malP was verified by sequence analysis (GATC Biotech).

The crude extract for MalP-His purification was obtained as described previously for the purification of RamA (59). The crude extract was applied to a His-Trap FF crude column (GE Healthcare) equilibrated with binding buffer (20 mM potassium phosphate, 0.5 M sodium chloride, 20 mM imidazole [pH 7.0]). The stepwise elution of proteins with imidazole was performed and monitored with an Äkta purifier system (GE Healthcare). Nonspecifically bound proteins were eluted at an imidazole concentration of 50 mM. The His-tagged MalP protein was eluted with a buffer containing 350 mM imidazole. Immediately after purification, EDTA (1 mM) was added to the fraction containing MalP-His. For further purification and removal of the imidazole, the fraction was first concentrated using Amicon Ultra centrifugal filter units (pore size, 30 kDa; Millipore) and then applied to a Superdex 200 10/300 GL gel filtration column (GE Healthcare) equilibrated with GF buffer (20 mM potassium phosphate, 50 mM sodium chloride [pH 7.0]). Elution was performed with a constant flow (0.5 ml/min). The fractions were collected and screened by Western blotting and activity analysis. For determination of

the molecular weight the retention volume of MalP-His was compared to that of standard proteins (HMW calibration kit; GE Healthcare).

Homologous overexpression of *malP* in *C. glutamicum*. For the homologous overexpression of *malP*, the plasmid pET16b-*malP* was digested using XbaI and BspEI. The resulting 2,630-bp fragment carrying the *malP* gene fused to the sequence encoding the His tag was purified via gel extraction and ligated into the XmaI- and XbaI-cut expression vector pXMJ19. The constructed vector, pXMJ19-*malP*, allows the IPTG-inducible expression of *malP* in *C. glutamicum*.

Protein analysis. Protein concentrations were determined by using the Roti-Nanoquant kit (Roth) with bovine serum albumin as the standard. SDS-PAGE was performed according to the method of Laemmli (60). Standard loading buffer (4×) contained 8% (wt/vol) SDS, 20% (vol/vol) glycerol, 10 mM EDTA, 100 mM Tris-HCl (pH 6.8), 2% (vol/vol) β-mercaptoethanol, and 1 mg of bromophenol blue/ml. Western blot experiments for detection of the His-tagged MalP protein were performed as previously described (43).

Enzyme assays. To determine chloramphenicol acetyltransferase (CAT) activity *C. glutamicum* cells were harvested, washed twice in 0.1 M Tris-HCl (pH 7.8), and resuspended in the same buffer containing 10 mM MgCl₂ and 1 mM EDTA. The specific CAT activity was determined as described by Schreiner et al. (61). The activities of MalP and MalQ were assayed in a final volume of 1 ml by spectrophotometric measurement of the variation in the NADP(H) concentration at 340 nm at 30°C. Cell extracts for the enzymatic measurements were prepared as follows. *C. glutamicum* cells were harvested at selected time points, washed twice in 0.1 M Tris-HCl (pH 7.4)–20 mM KCl–5 mM MgSO₄, and resuspended in 0.75 ml of the same buffer containing 0.1 mM EDTA, 2 mM dithiothreitol (DTT), and 10% (vol/vol) glycerol. The cell suspension was transferred to 2-ml screw-cap vials together with 250 mg of glass beads (150 to 212 μm; Sigma) and subjected five times for 30 s to mechanical disruption with a Percellys homogenizer (PecqLab) at 4°C, with intermittent cooling on ice for 5 min. After cell disruption, the glass beads and cellular debris were removed by centrifugation (10,000 × g, 4°C, 15 min). Afterward, the supernatant was centrifuged (55,000 × g, 1 h) to remove the membrane fraction. 4-α-Glucanotransferase activity (MalQ) activity was assayed by measuring the production of maltodextrins and glucose from maltotriose and maltose, as recently described (41).

Maltodextrin phosphorylase activity was measured in a continuous assay at 30°C with maltoheptaose as the substrate, essentially as described recently (41). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM MgCl₂, 2 mM NADP, 2 U of phosphoglucomutase from rabbit muscle (Sigma-Aldrich), and 2 U of glucose-6-phosphate dehydrogenase (Roche). After 2 min of preincubation, the assay was started by the addition of 5 mM maltoheptaose, and the reduction of NADP was monitored. For the determination of MalP substrate affinities, 5 to 100 mM maltotriose, 5 to 30 mM maltotetraose, 0.01 to 40 mM maltopentaose, 0.01 to 20 mM maltohexaose, 0.01 to 20 mM maltoheptaose, 0.025 to 2.3 mg of soluble starch (Sigma) ml⁻¹, and 2.3 mg of glycogen (Sigma) ml⁻¹ were used. The concentrations of ADP, AMP, and ADP-glucose tested as MalP effectors are indicated in Results.

RNA techniques. Isolation of total RNA from *C. glutamicum* cells was performed using the NucleoSpin RNAII kit as described by Wolf et al. (62). For Northern (RNA) hybridization, digoxigenin 11-dUTP-labeled gene specific antisense RNA probes were prepared from PCR products (generated with oligonucleotides listed in Table S1 in the supplemental material) carrying the T7 promoter by *in vitro* transcription (1 h, 37°C) using T7 RNA polymerase (MBI Fermentas). For hybridization, total RNA of *C. glutamicum* was separated on an agarose gel containing 17% (vol/vol) formaldehyde and transferred to a nylon membrane by using the VacuGene system (Pharmacia). RNA was cross-linked to the membrane by means of UV irradiation at 125 J cm⁻². Hybridization was carried out according to the digoxigenin application manual (Roche Applied Science). Slot blot experiments, densitometric detection, and signal quantification were performed as described previously (63, 64).

The transcriptional start site of *malP* was determined using a 5'/3'-RACE kit (Roche Diagnostics) according to the manufacturer's manual. First-strand cDNA was synthesized from 2 μg of total RNA using the gene-specific RACE-malP-SP1 primers (see Table S1 in the supplemental material). The subsequent PCRs were performed using the primer pair RACE-malP-SP1 and oligo(dT) anchor primer (the latter is included in the kit). The purified PCR product was ligated into plasmid pDrive (Qiagen), resulting in the recombinant plasmid pDrive-RACE-PR*malP*, which was sequenced. The transcriptional site was deduced from the sequences obtained.

Isolation and characterization of glycogen from *C. glutamicum* strains. For each preparation of α-glucans from *C. glutamicum* strains, four 200-ml cultures were cultivated in 1-liter baffled shake flasks in CGC minimal medium with 2% (wt/vol) maltose to an OD₆₀₀ of 8. Cells were harvested by centrifugation (Beckmann Avanti J-25 centrifuge; JLA 10.500 rotor; 5,000 × g, 4°C, 15 min) and washed twice with saline (0.9% [wt/vol] NaCl). The resulting pellets were resuspended in a total volume of 100 ml of double-distilled water. Cells were disrupted mechanically by continuously passing the cell suspension for 5 min at 4°C through an EmulsiFlex C5 high-pressure homogenizer (Avestin) at 15,000 lb/in². After removal of cellular debris by centrifugation (Eppendorf 5804R centrifuge; 10,000 × g, 4°C, 20 min), the proteins in the supernatant were precipitated by addition of 20% (wt/vol) trichloroacetic acid (TCA) and 1% (wt/vol) NaCl, incubation on ice for 1 h, and centrifugation for 30 min at 10,000 × g (Beckmann Avanti J-25 centrifuge; JA 25.50 rotor; 4°C). The α-glucans in the supernatant were precipitated by addition of 2 volumes of ethanol, incubation at 4°C for 8 h, and centrifugation for 30 min at 15,000 × g (Beckmann Avanti J-25 centrifuge; JLA 16.250 rotor; 4°C). The white precipitate was resuspended in 10 ml of double-distilled water, and the α-glucans were again precipitated by the addition of ethanol, incubation on ice, and centrifugation. The resulting precipitate was washed twice with 1 ml of diethyl ether and dried for at least 48 h in a desiccator, weighted, and then solved at a concentration of 10 mg ml⁻¹ in double-distilled water for further analysis.

For acid hydrolysis of α-glucans, 600 μl of 2 N HCl was added to 400 μl of α-glucan preparations, followed by incubation for 25 min at 95°C. The hydrolysis reaction was stopped by neutralization with 1 ml of 1.2 N NaOH, and the resulting solution was stored at 4°C for further analyses. The degradation of α-glucans to glucose by amyloglucosidase from *Aspergillus niger* (0.1 mg/ml, Fluka) was performed in 0.2 M sodium acetate buffer (pH 5.2) for 8 h at 56°C with 5 mg of the prepared α-glucans ml⁻¹. For the degradation of α-1,6-glycosidic linkages, 5 mg of the prepared α-glucans ml⁻¹ was incubated for 48 h with pullulanase from *Klebsiella pneumonia* (2 U ml⁻¹; Sigma-Aldrich) in 0.05 M ammonium acetate buffer (pH 5.0). Enzymatic reactions were stopped by boiling the samples for 10 min. Proteins from the preparation were removed by precipitation with 30% (wt/vol) TCA and subsequent washing with diethyl ether, as described previously (65).

For quantification of α-glucan degradation products, the bicinchoninic acid assay was used as described previously (42). Glucose solutions (0 to 25 μM) were used for the calibration. For qualitative analyses of glucans and degradation products, thin-layer chromatography (TLC) was performed. For this purpose, 5-μl samples were applied to the TLC plate (Adamant TLC glass plates, silica gel 60, 0.25 mm layer, 20 cm by 20 cm; Macherey & Nagel). Separation of carbohydrates was achieved by a solvent system consisting of 1-butanol, 2-propanol, ethanol, and water (3:2:3:2) in two consecutive ascents of 15 cm each. The carbohydrate spots were visualized by spraying with sulfuric acid solution (4% [wt/vol] H₂SO₄ in methanol) and heating the plates to 120°C for 5 min. Carbohydrates were identified by comparison to the migration of authentic standards, i.e., of glucose, maltose, maltotriose, maltoheptaose, and glycogen from oyster (all obtained from Sigma-Aldrich).

Microscopic imaging. For phase-contrast microscopy, 4 μl of a culture sample was placed on a microscope slide coated with a thin agarose (1%) layer and covered by a coverslip. Images were taken using a Zeiss

FIG 1 Specific MalP activity in cell extracts of *C. glutamicum* WT and *C. glutamicum* ΔglgAC (A) and specific CAT activities in *C. glutamicum* WT (pET2-PRmalP) and *C. glutamicum* ΔglgAC (pET2-PRmalP) (B). Cells for the assays were sampled in the exponential growth phase from cultivations in minimal medium with maltose (malt), glucose (glc), or acetate (ace) as the substrate at an OD₆₀₀ of 7. The data represent mean values of three independent determinations from at least three independent cultivations, and error bars represent the standard deviations. (C) Genetic map of the *cg1481* to *cg1479* locus in *C. glutamicum* WT. Genes are indicated as gray arrows, and predicted transcriptional terminators (TT1, TT2) are indicated as black bars. The transcriptional start site of *malP* (TS_{malP}) is indicated by an arrow.

Axiomager M1, equipped with a Zeiss AxioCam camera. Generally, an EC Plan-Neofluar 100×/1.3 oil Ph3 objective was used. Digital images were acquired with the AxioVision (Zeiss) software. Final image preparation was done in Adobe Photoshop 6.0 (Adobe Systems, Inc.).

Computational analysis. Identification of putative rho-independent transcriptional terminators was performed using TransTermHP (66), and mfold (67) was the software used for the calculation of their Δ*G*_{0'} values (free energy under standard conditions).

RESULTS

Carbon source-dependent changes of MalP activity and *malP* transcription. Previous RNA microarray studies of the *C. glutamicum* transcriptome showed a strong repression of *malP* transcription in cells cultivated on acetate compared to cells cultivated on glucose (45). To analyze probable substrate-dependent variations of MalP activity, *C. glutamicum* WT was cultivated in minimal medium containing maltose, glucose, or acetate (each at 2% [wt/vol]) as the sole substrate, and the cells were harvested in the exponential growth phase. As shown in Fig. 1A, the specific MalP activity was highest in cells cultivated on maltose (0.53 ± 0.05 U

mg protein⁻¹) and not significantly lower in cells from cultivations on glucose (0.46 ± 0.09 U mg protein⁻¹). In cells cultivated on acetate, a MalP activity of only 0.16 ± 0.02 U mg protein⁻¹ was detected, which is more than 3 times less than the specific activity determined in cells cultivated on maltose.

The *malP* (*cg1479*) gene is located within the *C. glutamicum* genome between the open reading frames *cg1478* and *cg1481*, which both encode short hypothetical proteins (Fig. 1C). The presence of rho-independent transcriptional terminators upstream (centered 170 bp upstream of the *malP* ATG start codon; Δ*G*_{0'}, 23.2 kcal mol⁻¹, TT1) (Fig. 1C) and downstream of the *malP* gene (centered 37 bp downstream of the TAG stop codon of *malP*; Δ*G*_{0'}, 27.1 kcal mol⁻¹, TT2) indicates a transcriptional organization of *malP* as a monocistronic operon. The transcriptional start site of *malP* (TS_{malP}) was determined by using 5' rapid amplification of cDNA ends (5'-RACE) with total RNA samples of maltose-grown *C. glutamicum* WT cells from the early and the late exponential growth phase. In three independent experiments, TS_{malP} was found to be the A residue of the annotated ATG start

codon. Upstream of TS_{malP} , we found the motif TAAACT, which is identical in four of six bases to the -10 consensus motif (TAC/TAAT) described for corynebacteria and which is identical to the -10 motif described for the promoters of *ppmA*, *pta* (P1), *clgR* (P2), *clpB* (P2), and *ilvN* (68, 69). To confirm the presence of a *malP*-specific promoter and to investigate transcriptional regulation of the *malP* gene, a transcriptional fusion between the putative *malP* promoter region and the promoterless chloramphenicol acetyltransferase (CAT) gene was constructed in the promoter probe vector pET2. The resulting plasmid, pET2-PR*malP*, was transformed into *C. glutamicum* WT, and CAT activities were determined in the plasmid-carrying strain during exponential growth in minimal medium containing maltose, glucose, or acetate (each at 2% [wt/vol]). Whereas the strain carrying the empty plasmid pET2 showed no CAT activity (<0.01 U mg protein $^{-1}$), a high CAT activity of 97.70 ± 16.90 mU mg protein $^{-1}$ was detected for cells of *C. glutamicum* (pET2-PR*malP*) grown on maltose (Fig. 1B). CAT activity in *C. glutamicum* (pET2-PR*malP*) cells cultivated on glucose was slightly, albeit not significantly, lower (73.76 ± 13.81 mU mg protein $^{-1}$). In accordance with the low MalP activity in acetate-grown cells observed here and data from previous RNA microarray studies, very low CAT activities of 4.53 ± 0.81 mU mg protein $^{-1}$ were detected in *C. glutamicum* (pET2-PR*malP*) cells cultivated on acetate. Taken together, these data clearly show the carbon source-dependent regulation of *malP* transcription in *C. glutamicum*.

MalP is required in the late exponential growth phase for normal growth on maltose. As previously reported for cultivations with maltose as sole substrate, the growth rate of *C. glutamicum* $\Delta malP$ decreased at the mid-exponential-growth phase (after 6 to 7 h of cultivation) from initially 0.33 ± 0.04 h $^{-1}$ to 0.05 ± 0.02 h $^{-1}$, whereas the growth rate of the parental strain *C. glutamicum* WT did not change at this time point (41). Introduction of the plasmid pXMJ19-*malP*, which carries the *malP* gene under the control of the IPTG-inducible promoter *ptac*, restored WT-like growth of *C. glutamicum* $\Delta malP$ on maltose: no differences in growth between the strain *C. glutamicum* $\Delta malP$ (pXMJ19-*malP*) and *C. glutamicum* WT during cultivation in minimal medium with 2% maltose were observed (see Fig. S1A in the supplemental material) [growth rates of 0.34 ± 0.06 h $^{-1}$ and 0.35 ± 0.04 h $^{-1}$ were determined for *C. glutamicum* $\Delta malP$ (pXMJ19-*malP*) and *C. glutamicum* WT, respectively]. In contrast, the growth rate of *C. glutamicum* $\Delta malP$ (pXMJ19), which carries the empty plasmid, decreased at the mid-exponential phase (after 6 to 7 h of cultivation) from initially 0.32 ± 0.05 h $^{-1}$ to 0.07 ± 0.03 h $^{-1}$. The specific MalP activity in *C. glutamicum* $\Delta malP$ (pXMJ19-*malP*) cultivated on maltose in the presence of 0.1 mM IPTG was nearly identical to the activity determined for *C. glutamicum* WT (Fig. 2B) [specific activities of 0.44 ± 0.16 U mg protein $^{-1}$] and 0.46 ± 0.10 U mg protein $^{-1}$ were determined for *C. glutamicum* $\Delta malP$ (pXMJ19-*malP*) and *C. glutamicum* WT, respectively]; low MalP activities were observed with the empty-vector control strain *C. glutamicum* $\Delta malP$ (pXMJ19) (0.02 ± 0.01 U mg protein $^{-1}$) (Fig. S1B), as well as for *C. glutamicum* $\Delta malP$ (0.03 ± 0.02 U mg protein $^{-1}$). In addition, cells of *C. glutamicum* $\Delta malP$ (pXMJ19-*malP*) showed the typical cell shape of *C. glutamicum* WT cells in cultivations on maltose (see Fig. S1C in the supplemental material), whereas cells of *C. glutamicum* $\Delta malP$ (pXMJ19) were enlarged and deformed as had been previously reported for *C. glutamicum* $\Delta malP$ (41). Taken together, these results confirm that

FIG 2 (A) Growth (circles), specific MalP and MalQ activities (black and gray bars, respectively), and substrate utilization (diamonds) in the course of cultivations of *C. glutamicum* WT in minimal medium as 1-liter batch cultures with 2% (wt/vol) maltose as the substrate in a stirred, pH-controlled bioreactor. The data represent mean values of three independent determinations from two independent cultivations, and error bars represent the standard deviations. (B) Analysis of *malP* transcription by Northern blotting in RNA samples from cells sampled at different time points from the bioreactor cultivation of *C. glutamicum* WT on maltose. For the Northern blot analysis, RNA was separated by electrophoresis and probed with a *malP*-specific RNA probe; the sizes of RNA fragments determined with a RiboRuler high-range RNA ladder (Fermentas) are shown on the right.

the *malP*-encoded enzyme is the main α -glucan phosphorylase of *C. glutamicum*, which is required for normal growth in the late exponential growth phase during cultivation on maltose.

MalP activity and *malP* transcription are increased at mid-exponential phase during cultivation on maltose. To have a closer look at the physiological function of MalP at the late exponential phase, we studied MalP activity, *malP* expression, substrate consumption, and glycogen content of *C. glutamicum* WT in maltose-minimal medium in a 1-liter batch cultivation using a controlled bioreactor providing a constant pH of 7.0 and a constant oxygen concentration of 30% saturation. *C. glutamicum* WT grew at a growth rate of 0.34 ± 0.02 h $^{-1}$ and reached a final OD₆₀₀ of 44.3 ± 2.2 after 11 h of cultivation, when the initially provided 2% (wt/vol) maltose was completely consumed (Fig. 2A). The glycogen (55.1 ± 3.8 mg glucose g of cells $^{-1}$ [dry weight]) accumulated at the early exponential growth phase was degraded in the progression of the exponential growth phase. After 10 h of cultivation, just before the stationary phase set in, only low concentra-

tions of glycogen (6.6 ± 0.4 mg glucose g of cells⁻¹ [dry weight]) (see Fig. S2A in the supplemental material) were present in the cells. A nearly constant MalP activity between 0.21 ± 0.01 and 0.27 ± 0.01 U mg protein⁻¹ was detected in the first 7 h of the cultivation (Fig. 2A). In the late exponential growth phase, MalP activity increased to 0.95 ± 0.02 U mg protein⁻¹ before it finally decreased to 0.47 ± 0.01 U mg protein⁻¹, which is still twice as much of MalP activity observed in samples from the early exponential growth phase. Transcription of *malP* in the course of cultivation on maltose was analyzed using Northern blots (Fig. 2B). The hybridization with a *malP*-specific RNA probe revealed two signals. The fragment size of 2.5 kbp of one signal corresponds well to the size of the *malP* gene (2,388 bp) and thus confirms the proposed monocistronic organization. The second signal corresponds to a fragment size of ~1,400 nucleotides and is probably a degradation product of the *malP* transcript, since the intensities of the two signals detected in the Northern blot analysis of *malP* transcription showed identical variations. The changes of *malP* signal intensities in the course of time observed in the Northern blot (Fig. 2B) and in additional slot blot experiments (see Fig. S2B in the supplemental material) were evaluated by densitometry. These analyses revealed that *malP* transcript levels were also significantly increased after 7 h of cultivation just at the beginning of the mid-exponential growth phase (see Fig. S2A in the supplemental material). Direct at the onset of the stationary-phase *malP* transcript levels decreased drastically; thus, no *malP* transcripts were detected in samples after 10 h of cultivation (Fig. 2 and see Fig. S2B in the supplemental material). This pattern of *malP* transcription and expression led us to investigate the transcription and expression of further genes involved in maltose and maltodextrin metabolism such as *malQ* and the genes *musK*, *musE*, and *musG*, the latter three genes encoding essential components of the *C. glutamicum* maltose uptake system MusEFGK_I (38). The *malQ* transcript levels (see Fig. S3 in the supplemental material) were also steady in the early exponential growth phase and increased about twice in the late exponential growth phase. As reported here for *malP*, *malQ* transcription was immediately absent at the onset of the stationary growth phase after ~10 h of cultivation. Analyses of the specific MalQ enzyme activities in the course of the cultivation also revealed the pattern described above for MalP: in the first 7 h of cultivation, constant MalQ activities were observed, ranging between 0.31 ± 0.01 and 0.41 ± 0.02 U mg protein⁻¹ (Fig. 2A), then increased to 0.97 ± 0.06 U mg protein⁻¹ and remained at this level for 3 h, before decreasing again to 0.63 ± 0.05 U mg protein⁻¹ just at the onset of the stationary growth phase. In contrast, transcripts of the genes *musK*, *musE*, and *musG* were still present in high abundance in samples taken after 10 h of cultivation (see Fig. S3 in the supplemental material). The transcript levels of these three genes decreased only slowly within the stationary phase; in samples taken 11 h after the start of the cultivation, transcripts of *musK*, *musE*, and *musG* were still detected (albeit in smaller amounts) (see Fig. S3 in the supplemental material), whereas no *malP* and *malQ* transcripts were observed in RNA samples from the late stationary phase (Fig. 2B; see Fig. S2B and Fig. S3 in the supplemental material).

Taken together, these results show that *malP* transcription and MalP activity increase in the late exponential growth phase and are reduced or even absent directly at the start of the stationary growth phase. These changes coincide with the occurrence of the growth defect in the MalP-deficient *C. glutamicum* strain at the

late exponential growth phase during cultivations on maltose and therefore indicate the importance of MalP for *C. glutamicum* in this growth phase.

Glycogen formation does not influence *malP* expression.

Changes in *malP* transcription and expression in the course of cultivations and in the dependence of the carbon source might be caused in *C. glutamicum* by endogenous induction, as described for *E. coli* for the control of the *malPQ* operon. Transcription of *malPQ* is controlled in *E. coli* by the transcriptional activator MalT, which depends on the presence of maltotriose as an effector molecule for its activity (24, 70, 71). Maltotriose is formed in the course of maltose utilization, as well as in the course of glycogen degradation; thus, both metabolic processes contribute to the induction of *malPQ* transcription in *E. coli* (25). In contrast to cultivations on glucose and maltose, *C. glutamicum* accumulates only small amounts of glycogen in cultivations on acetate (35, 41). Hence, the observed substrate-dependent changes of *malP* transcription and MalP activity might be caused indirectly by the lack of endogenous induction from glycogen derived maltotriose in cells cultivated on acetate. To distinguish between endogenous induction and substrate-dependent control of *malP* expression, analyses of *malP* transcription and MalP activity in a glycogen synthesis-deficient *C. glutamicum* strain might be useful. Indeed, inactivation of *glgC* in *C. glutamicum* WT (resulting in *C. glutamicum* strain IMglgC) (35) abolished glycogen synthesis in cultivations on glucose. However, we observed formation of significant amounts of a high-molecular-weight, putative glucose-polymer in cultivations of *C. glutamicum* IMglgC on maltose (see Fig. S4 in the supplemental material). To further investigate the accumulation of this likely glucose polymer, the deletion mutant *C. glutamicum* Δ glgAC was constructed, which lacks the genes for both ADP-glucose pyrophosphorylase and glycogen synthase. Whereas only residual amounts of glycogen were detected in cultivations on glucose (Fig. 3A), *C. glutamicum* Δ glgAC also accumulated up to 67.7 ± 12.7 mg glucose g of cells⁻¹ (dry weight) of a glucose polymer in cultivations on maltose (Fig. 3B). The compositions of the isolated polymer from *C. glutamicum* Δ glgAC and of glycogen from maltose-grown *C. glutamicum* WT were analyzed using a combination of enzymatic digestion and TLC (Fig. 3C) or quantification of the reducing ends formed during digestion (see Table S2 in the supplemental material). As shown in Fig. 3C, the isolated polymers from both strains were completely degraded to glucose by the action of the amyloglucosidase, which hydrolyzes both α -1,4- and α -1,6-glycosidic linkages. Incubation of the isolated glucose polymers with pullulanase, which exclusively cleaves α -1,6-glycosidic bonds, led to the formation of various degradation products. Even after prolonged incubation with pullulanase, the isolated polymers of both strains were not completely degraded. Taken together, these results show that the isolated polymers of both strains consist of α -1,4- and α -1,6-glycosidic linked glucose molecules. Since the quantitative analysis of the composition also showed only minor differences between the glucose polymers of *C. glutamicum* WT and *C. glutamicum* Δ glgAC (see Table S2 in the supplemental material), it can be concluded that both strains form a glycogen-like glucose polymer in the course of cultivations on maltose, which would be a likely source of molecules for the supposed endogenous induction of *malP* expression in *C. glutamicum*. However, analysis of MalP activity in glucose- and maltose-grown cells of *C. glutamicum* Δ glgAC revealed only a slightly lower activity in cells grown on glucose (0.34 ± 0.05 U mg

FIG 3 Growth (symbols) and glycogen content (bars) of *C. glutamicum* WT (circles and dark gray bars) and *C. glutamicum* ΔglgAC (triangles and light gray bars) in the course of cultivation in minimal medium with 2% (wt/vol) glucose (A) or 2% (wt/vol) maltose (B). For growth, one representative data set of at least three independent cultivations is shown; the results of each of the cultivations were comparable. Glycogen concentrations are mean values from three independent cultivations, and error bars represent the standard deviations. (C) TLC analysis of isolated glucan and carbohydrates obtained by its degradation with amyloglucosidase or pullulanase derived from *C. glutamicum* WT and *C. glutamicum* ΔglgAC cells cultivated on maltose. The standard solution contained glucose, maltose, maltotriose, maltoheptaose, and glycogen (each at 5 μg ml⁻¹).

protein⁻¹) than in cells from cultivations on maltose (0.46 ± 0.06 U mg protein⁻¹) (Fig. 1A). In accordance with these findings, the *malP* promoter activity was also only slightly affected by the loss of glycogen accumulation, and no significant changes of CAT activities were detected in maltose- and glucose-grown cells of *C. glutamicum* ΔglgAC (pET2-PR*malP*) (83.46 ± 7.28 mU mg protein⁻¹) and 56.12 ± 5.71 mU mg protein⁻¹, respectively) compared to the CAT activities observed in *C. glutamicum* WT (pET2-PR*malP*) (Fig. 1B). In general, the MalP and CAT activities were only slightly lower in *C. glutamicum* ΔglgAC than in *C. glutamicum* WT. These results indicate that endogenous induction

by glycogen degradation products is not significantly contributing to the control of *malP* transcription in *C. glutamicum*.
Analysis of substrate specificity of MalP. Despite the high sequence identity to the well-characterized starch phosphorylase CcStP from *C. callunae*, the *cg1479* gene product of *C. glutamicum* was designated MalP because deletion of the gene negatively affected growth on maltose (41). However, thus far, the substrate spectrum of MalP from *C. glutamicum* has not been analyzed. For this purpose, MalP was synthesized as an N-terminal His-tagged fusion protein in *E. coli* BL21(DE3) and purified using ultracentrifugation, affinity chromatography, and size exclusion chroma-

FIG 4 (A) Purification of recombinant *C. glutamicum* MalP from *E. coli* BL21(DE3) (pET16b-malP). SDS-polyacrylamide gel electrophoresis analysis of crude extracts (CE), supernatant after ultracentrifugation (UC), the Ni²⁺-affinity chromatography elution fraction (AC), a concentrated sample after ultrafiltration (UF), the main fraction from gel filtration (SEC), and a PAGE ruler prestained protein ladder (MBI Fermentas) containing proteins of indicated molecular mass (lane M) was performed. (B) Molecular mass of purified *C. glutamicum* MalP determined by gel filtration. The ratio of the V_e (elution volume) to the V_o (void volume measured by dextran blue) of standard proteins (ferritin, aldolase, conalbumin, and ovalbumin) was plotted against the molecular mass, as seen in the inset. From this plot, the native molecular mass of MalP was calculated. The activity of purified *C. glutamicum* MalP at different concentrations of maltopentaose (0.001 to 40 mM) (C), maltohexaose (0.01 to 20 mM) (D), and maltoheptaose (0.01 to 20 mM) (E) was plotted. The data represent mean values of at least two independent determinations from two independent MalP purifications and were fitted according to the Michaelis-Menten equation, and the error bars represent the standard deviations.

tography. SDS-PAGE analysis of the products of each purification step finally revealed a single protein of ~80 kDa (Fig. 4A), which could also be detected by Western blotting with antibodies binding to the N-terminal His tag (data not shown). Analysis of the elution profile from the size exclusion chromatography performed in the course of MalP purification revealed an oligomeric status. MalP eluted as a homogenous peak at a volume of 12.4 ml, corresponding to a molecular mass of 179 ± 5 kDa, compared to standard proteins (Fig. 4B). This result indicates that the native MalP represents a homodimer, since it was also shown for the glucan phosphorylases CcStP (52) and MalP from *E. coli* (9). The purified MalP protein was fully active for up to 4 days when stored on ice. Addition of reducing agents such as DTT or freezing of MalP preparations led to a significant loss of enzyme activity.

Purified MalP showed only very low activity with both maltotriose and maltotetraose as the substrate (data not shown). Anal-

ysis of MalP activity with various concentrations of maltopentaose (0.01 to 40 mM) revealed a saturation kinetic, with a K_m of 0.68 ± 0.25 mM and a V_{max} of 2.02 ± 0.11 U mg protein⁻¹ (Fig. 4C). A similar V_{max} of 1.91 ± 0.07 U mg protein⁻¹ and a slightly lower K_m of 0.33 ± 0.07 mM were determined when maltohexaose (0.01 to 20 mM) was used as a substrate for MalP (Fig. 4D). With maltoheptaose as the substrate (0.01 to 20 mM) for purified MalP a significantly higher V_{max} of 6.59 ± 0.22 U mg protein⁻¹ was observed (Fig. 4E), albeit the affinity with maltoheptaose ($K_m = 0.63 \pm 0.08$ mM) was lower than the affinity of MalP for maltohexaose. Also, with starch as a substrate (0.023 to 2.3 mg ml⁻¹), a saturation kinetic for purified *C. glutamicum* MalP was observed with a maximal activity of 5.54 ± 0.27 U mg protein⁻¹ (see Fig. S5 in the supplemental material), a K_m value of 0.16 ± 0.03 mg ml⁻¹ starch was calculated. When glycogen was used instead of starch as the substrate for MalP, the activity not significantly increased from

5.41 ± 0.74 U mg protein⁻¹ to 6.03 ± 0.42 U mg protein⁻¹ (both substrates were used at a concentration of 2.3 mg ml⁻¹). Taken together, no preference of MalP for the degradation of either large, branched α -glucans such as glycogen and starch or short, linear glucans such as maltoheptaose was observed. Thus, classification of MalP as maltodextrin, glycogen, or starch phosphorylase based on its substrate preference is not possible.

Competitive inhibition of *C. glutamicum* MalP by ADP-glucose. Functional classification of bacterial α -glucan phosphorylases as glycogen or as maltodextrin phosphorylase based on substrate specificity is often not possible, and so the mode of regulation is used as a criterion (15). As shown above, we observed transcriptional regulation of the *C. glutamicum* malP, a typical criterion for classification of the gene product as a MalP. However, we also analyzed the effects of the metabolites AMP, ADP, and ADP-glucose on MalP activity. The three metabolites have been described as allosteric activators or inhibitors of GlgP but not of MalP enzymes. With 10 mM maltoheptaose as the substrate, the corynebacterial MalP activity of initially 6.05 ± 0.62 U mg protein⁻¹ was reduced to 3.49 ± 0.45 U mg protein⁻¹ and 3.70 ± 0.51 U mg protein⁻¹ upon the addition of 10 mM AMP or 10 mM ADP, respectively. The addition of ADP-glucose nearly abolished MalP activity; only a residual MalP activity of 0.14 ± 0.03 U mg protein⁻¹ was observed when 10 mM ADP-glucose was added to the assay. Inhibition of MalP by ADP-glucose was further investigated. As shown in Fig. S6 in the supplemental material, increasing amounts of ADP-glucose efficiently inhibited MalP activity with maltoheptaose (5 mM), as well as starch (1.8 mg ml⁻¹), as the substrate. The addition of 0.4 mM ADP-glucose caused a 70% reduction in MalP activity from 5.79 ± 0.56 U mg protein⁻¹ to 1.75 ± 0.17 U mg protein⁻¹ with maltoheptaose as the substrate and from 4.73 ± 0.17 U mg protein⁻¹ to 1.48 ± 0.77 U mg protein⁻¹ with starch as the substrate. No effects of ADP-glucose addition on the activities of Pgm (from rabbit muscle) and glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) used in the MalP assay were observed (data not shown). Figure 5 shows a reciprocal plot of MalP velocity versus maltoheptaose concentration in the presence of 0.1 mM, 0.25 mM, and no ADP-glucose. ADP-glucose caused an increase in the K_m of MalP for maltoheptaose from initially 0.63 ± 0.22 mM to 2.47 ± 0.70 mM in the presence of 0.1 mM ADP-glucose and to 5.67 ± 1.81 mM in the presence of 0.25 mM ADP-glucose. Since the affinity for maltoheptaose and not the maximal velocity of MalP was affected by ADP-glucose addition (Fig. 5), it can be concluded that MalP of *C. glutamicum* is competitively inhibited by ADP-glucose. Taking into account that ADP-glucose is an intermediate of glycogen synthesis, the inhibition of MalP by ADP-glucose suggests that MalP functions in glycogen degradation as glycogen phosphorylase. However, this conclusion contradicts the aforementioned classification of the glucan phosphorylase MalP as a maltodextrin phosphorylase, deduced from the finding of carbon source-dependent transcriptional regulation of malP. Thus, *C. glutamicum* MalP is the first example of a bacterial α -glucan phosphorylase, which cannot be easily classified based on the mode of its control.

DISCUSSION

Coordination of synthesis and degradation of storage compounds such as glycogen or starch requires efficient control mechanisms that allow the integration of physiological signals. Considering

FIG 5 Effect of ADP-glucose on MalP activity. A Lineweaver-Burk plot shows the MalP activity at various maltoheptaose concentrations (1 to 10 mM) and different fixed ADP-glucose concentrations: 0 mM (circles), 0.1 mM (squares), and 0.25 mM (triangles). The data represent the mean values of two independent determinations from two independent MalP purifications. Non-linear least-squares estimation was performed for all data sets, yielding $r = 0.993$, $r = 0.972$, and $r = 0.978$, respectively.

that in bacteria such as *E. coli* and *C. glutamicum* substrate specificities of enzymes for the degradation of glycogen and for maltose utilization overlap, these two metabolic processes have to be synchronized. *E. coli* possesses the two α -glucan phosphorylases GlgP and MalP which both can degrade glycogen as well as the maltodextrins formed in the course of maltose metabolism (4, 15). Coordination of the two glucan phosphorylases GlgP and MalP in *E. coli* for glycogen and maltose metabolism, respectively, is achieved primarily by the organization of the respective genes in operons with genes for glycogen metabolism (*glgCAP*) or maltose utilization (*malPQ*) (29, 72) and also, as in the case of MalP, by transcriptional control of its expression (23, 70, 73, 74), or as reported for GlgP, by allosteric control of its activity (15, 18). In contrast to the situation in *E. coli*, the genes for glycogen and maltose metabolism are not organized in operons in *C. glutamicum* and are not well distributed in the genome. Physiological studies also revealed that in *C. glutamicum*, glycogen synthesis and degradation have to be coordinated efficiently throughout cultivation since the glycogen level is set by the concerted action of glycogen-synthesizing and -degrading enzymes (43). The finding that activity of *C. glutamicum* MalP is controlled by competitive inhibition by the glycogen synthesis intermediate ADP-glucose is the first mechanism for the coordination of glycogen synthesis and degradation observed in *C. glutamicum*. This mode of MalP activity control causes the fast degradation of glycogen in response to limitations of the glycogen synthesis. By this means, fluctuations of the central metabolism intermediates glucose-1-phosphate and glucose-6-phosphate are probably avoided, which reflects the proposed function of glycogen as a carbon capacitor in *C. glutamicum* (40, 43).

Carbon source-dependent regulation of malP transcription in *C. glutamicum* leads to high levels of malP transcripts and MalP activity in cultivations on glucose and maltose when glycogen is accumulated and to small amounts of malP transcripts and activity in cultivations on acetate when glycogen accumulation does

not take place. In a similar manner, transcription of the genes *glgC*, *glgA*, and *glgB* for glycogen synthesis is induced in cultivations with glucose and repressed in cultivations with acetate (42, 44). The two global regulators RamA and RamB are involved in the control of *glgC* and/or *glgA* transcription; however, the substrate-dependent transcriptional control of these two genes is brought about by an as-yet-unknown regulator (44). Since the lack of glycogen accumulation in glucose-grown *C. glutamicum* Δ *glgAC* cells did not affect *malP* promoter activity and endogenous induction, it could be excluded as a stimulus for *malP* transcription. Thus, it can be assumed that a general mechanism for the coordinated, carbon source-dependent transcription of genes for glycogen synthesis and degradation exists in *C. glutamicum*. In addition to these carbon source-dependent variations, we observed growth phase-dependent changes of *malP* promoter activities. In maltose-grown *C. glutamicum* cells, the *malP* transcript level and the MalP activity increased at the end of the exponential growth phase before the initially provided substrate became limiting. In a similar way, *malQ* transcript levels and MalQ activity varied in the course of cultivations on maltose, whereas no similar variations were observed for the genes *musK*, *musE*, and *musF*, which encode components of the maltose uptake system. In *E. coli*, both MalP and MalQ contribute to glycogen degradation (25), so the increased amounts of MalP and MalQ in the late-exponential growth phase in *C. glutamicum* might indicate a change in the balance of enzymes for glycogen synthesis and degradation, causing the degradation of accumulated glycogen before the entry of the stationary growth phase (35, 43). The stimulus for this change in the balance of glycogen synthesis and degradation indicated by increased transcription of *malP* seems to be interrelated with substrate availability. However, *malP* transcript levels, as well as MalP activities, increased in the bioreactor experiments already at a residual maltose concentration of ~ 32 mM (Fig. 2A), which is 1,550-fold higher than the maltose concentration of ~ 20 μ M required for the maltose uptake system MusEFGK₂I to work at its maximal velocity (38). Thus, the increase in *malP* transcription sets actually in before the substrate becomes limiting and it seems that *C. glutamicum* anticipates the start of substrate limitation and starts to change its metabolism in preparation for the stationary growth phase. Factors such as the accumulation of by-products and/or cell density might be possible triggers for late-exponential-phase *malP* transcriptional regulation in *C. glutamicum*, and yet signals and mechanisms for the recognition of upcoming limitations of glycolytic substrates, as well as the identity of regulators for *malP* transcriptional control, remain to be investigated.

In the course of the characterization of the transcriptional control of *malP*, we observed the formation of glycogen in the *glgC*- and *glgA*-deficient strain *C. glutamicum* Δ *glgAC* when cultivated on maltose. The glycogen synthesis pathway via the *glgC*-encoded ADP-glucose pyrophosphorylase and the *glgA*-encoded glycogen synthase is also the major route for glycogen synthesis in *E. coli* (2, 75). However, also for *glgC*- and/or *glgA*-deficient *E. coli* strains, glycogen synthesis has been described (76–78): apart from the ADP-glucose pyrophosphorylase, additional, unknown enzymes for ADP-glucose synthesis are present in *E. coli*. Thus, *glgC*-deficient *E. coli* strains still synthesize small amounts of glycogen in cells grown on glucose (76, 77). Deletion of *glgA* in *E. coli* abolishes glycogen synthesis in cultivations on glucose; however, *glgA*-deficient *E. coli* cells growing on maltose synthesize glycogen-like α -glucans from long maltodextrins formed by MalQ (78). Also,

mycobacteria such as *Mycobacterium tuberculosis* and *M. smegmatis* synthesize α -glucans not only via the well-known GlgC-GlgA pathway but also by two further pathways (79). The first alternative pathway proceeds via the *rv3032*-encoded glycosyltransferase in *M. tuberculosis* and is mainly involved in the formation of methylglucose lipopolysaccharides (80, 81). The second alternative pathway for α -glucan formation in mycobacteria proceeds via the consecutive action of the maltokinase Pep1 and the maltosyltransferase GlgE (82, 83). The presence of alternative sources of ADP-glucose is not relevant for the formation of glycogen in *C. glutamicum* Δ *glgAC* cultivated on maltose observed here, since this strain lacks the *glgA*-encoded glycogen synthase. In addition to *malQ*, genes for the alternative α -glucan synthesis pathway via Pep1 and GlgE are also present in *C. glutamicum*, whereas no gene with a significant similarity to *rv3032* was found in the genome (37). Glycogen synthesis in *glgC*- and/or *glgA*-deficient *C. glutamicum* strains has exclusively been detected in cultivations on maltose; no accumulation of glycogen was detected in cultivations of *C. glutamicum* IMglgC and *C. glutamicum* Δ *glgAC* on glucose (the present study), ribose, and fructose (data not shown) or in cultivations of *glgA*-mutant strains on sucrose (34). Since maltose is used as a precursor by both the alternative glycogen synthesis pathway, via maltokinase and maltosyltransferase, and the MalQ pathway, the GlgC- and GlgA-independent formation of glycogen in *C. glutamicum* cultivations on maltose observed here could be attributed to either one of the two pathways. However, the contribution of these two pathways to glycogen synthesis remains to be investigated.

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