Regulation of the glucose-specific phosphotransferase system (PTS) of Staphylococcus carnosus by the antiterminator protein GlcT

Igor Knezevic,1 Steffi Bachem,2 Albert Sickmann,3 Helmut E. Meyer,3 Jörg Stülke2 and Wolfgang Hengstenberg1

Author for correspondence: Wolfgang Hengstenberg. Tel: +49 234 3224247. Fax: +49 234 3214620. e-mail: wolfgang.hengstenberg@ruhr-uni-bochum.de

The ptsG operon of Staphylococcus carnosus consists of two adjacent genes, glcA and glcB, encoding glucose- and glucoside-specific enzymes II, respectively, the sugar permeases of the phosphoenolpyruvate-dependent phosphotransferase system (PTS). The expression of the ptsG operon is glucose-inducible. Putative RAT (ribonucleic antiterminator) and terminator sequences localized in the promoter region of glcA suggest regulation via antitermination. The glcT gene was cloned and the putative antiterminator protein GlcT was purified. Activity of this protein was demonstrated in vivo in Escherichia coli and Bacillus subtilis. In vitro studies led to the assumption that phosphoenolpyruvate-dependent phosphorylation of residue His105 via the general PTS components enzyme I and HPr facilitates dimerization of GlcT and consequently activation. Because of the high similarity of the two ptsG-RAT sequences of B. subtilis and S. carnosus, in vivo studies were performed in B. subtilis. These indicated that GlcT of S. carnosus is able to recognize ptsG-RAT sequences of B. subtilis and to cause antitermination. The specific interaction between B. subtilis ptsG-RAT and S. carnosus GlcT demonstrated by surface plasmon resonance suggests that only the dimer of GlcT binds to the RAT sequence. HPr-dependent phosphorylation of GlcT facilitates dimer formation and may be a control device for the proper function of the general PTS components enzyme I and HPr necessary for glucose uptake and phosphorylation by the corresponding enzyme II.

Keywords: antitermination, glucose-specific phosphotransferase system, regulation, Staphylococcus carnosus, protein phosphorylation

INTRODUCTION

Gene expression in bacteria is generally regulated at the transcriptional level. Various mechanisms control the initiation of transcription or the length of transcript which leads to truncated mRNA. Antitermination determines the length of transcript via antiterminator/terminator sequences (RAT/Tₜ sequence) which are located in the leader or intercistronic regions of an operon. According to the mechanism, two different variants can be described. The first form is termed processive antitermination and describes the modification of the RNA polymerase to a terminator-resistant complex. Such a complex is able to transcribe through multiple transcriptional ρ-dependent terminators. This kind of antitermination is most often found in viral development (Greenblatt et al., 1993).

The second type is so-called non-processive antitermination. In this case antitermination is mediated by antiterminator proteins interacting with nascent mRNA sequences. These ribonucleic antiterminator (RAT) sequences overlap ρ-independent transcription terminators. A complex of the RAT with the antiterminator protein stabilizes the secondary structure of
the RAT and thus prevents an alternative formation of the terminator loop (Aymerich & Steinmetz, 1992; Arnaud et al., 1992).

Various genes and operons are controlled by antitermination, including rRNA synthesis and production of proteins involved in carbohydrate transport and utilization (Landick et al., 1990; Yanofsky et al., 1987; Rutberg, 1997).

Antitermination of catabolic operons appears to be substrate-induced via reversible phosphorylation of the antiterminator proteins. The transmitters are components of the phosphoenolpyruvate-dependent systems (PTS) as well as by the carbohydrate-specific components (enzyme II) of the PTS (Schnetz & Rak, 1988, 1990; Arnaud et al., 1996). The antitermination systems of several catabolic operons appears to be involved in the metabolism of sugars like glucose, sucrose and β-glucosides are assigned to the bgl-sac family based on sequence similarities with regard to antiterminator proteins and RAT sequences (Rutberg, 1997; Stülke et al., 1998). The antiterminator proteins of this family are composed of three structural domains. The N-terminal residues are involved in binding the RAT sequence (Manival et al., 1997). The two other domains, called PRD-I and PRD-II (PTS regulatory domain), contain two similar regions which are phosphorylated at conserved histidine residues by components of the PTS (Stülke et al., 1998). Genetic and biochemical evidence points to a modulation of the binding of the antiterminator to its corresponding RAT sequence by PTS components (Amster-Choder & Wright, 1992, 1997; Arnaud et al., 1992).

In this study we show in vitro and in vivo studies which provide evidence for regulation of the ptsG operon of Staphylococcus carnosus via antitermination. GlcT was cloned and the antiterminator protein GlcT was purified. A PTS-mediated phosphorylation and its influence on quaternary structure was observed. Because of the high similarities to the ptsG antitermination system of Bacillus subtilis, the complementation between these two systems was investigated in an in vivo study in B. subtilis and analysis of RNA-binding activity through surface plasmon resonance.

METHODS

Chemicals. Oligonucleotides, yeast extract and tryptone were purchased from Life Technologies; trypsin (sequencing grade) was purchased from Promega; all other reagents were the purest grades available.

Bacterial strains and growth conditions. Escherichia coli DH5α and XL-1 Blue (Sambrook et al., 1989), and Staphylococcus carnosus TM300 were used for cloning experiments. E. coli BL21 (DE3) (Sambrook et al., 1989) was used for expression of S. carnosus GlcT. B. subtilis GlcT was expressed in E. coli FT1. E. coli was grown in TBY medium (10 g tryptone l−1, 5 g yeast extract l−1, 5 g NaCl l−1, 50 µg ampicillin l−1).

B. subtilis strains QBS448 [trpC2 amyE[ΔLA ptsG−lacZ aphA3)]; Stülke et al., 1997] and GPl09 [trpC2 ΔglcT8 amyE[ΔLA ptsG−lacZ aphA3]; Bachem & Stülke, 1998] were grown in SP or CSE medium (Faires et al., 1999). Media were supplemented with carbon sources as indicated.

DNA manipulations. All manipulations with recombinant DNA were carried out by standard procedures (Sambrook et al., 1989). Restriction enzymes and T4 ligase used in recombinant DNA experiments were used according to the specifications of the supplier. A fragment was amplified from agarose gels with the Nucleo Spin Extract kit from Macherey & Nagel. Genomic DNA from S. carnosus TM300 was purified using the Genomic DNA Kit from Qiagen. DNA sequences were determined with the ALF DNA Sequencer (Pharmacia; Kristensen et al., 1988).

Cloning of the glcT gene. To generate a digoxigenin (DIG)-labelled fragment, oligonucleotide primers were designed according to the partial ORF upstream of the gene glcA. Primers glcT1 (5′-CCAGAAAAGATGAGTGG-3′) and glcT2 (5′-GGTGTTGTAAGTGTAGT-3′) correspond to amino acids 1–5 and 132–138 of the truncated ORF localized on pUC18-7k (see below), respectively. These primers were used for PCR with construct pUC18-7k which bears the partial ORF of the putative glcT as template (Christiansen & Hengstenberg, 1996, 1999). The PCR mixture (50 µl) contained 200 ng pUC18-7k, 50 pmol each primer, DIG-dNTP mix (Boehringer Mannheim) and 2.5 U Taq polymerase (Life Technologies) in the recommended buffer.

Genomic DNA was prepared from S. carnosus TM300 by using the genic isolation kit from Qiagen. Southern blots of digested genomic DNA were probed with DIG-labelled DNA (see above) using the Genius kit from Boehringer Mannheim. A subgenomic S. carnosus library was constructed using a size-fractionated (2–3 kb) pool of BarYI-digested chromosomal DNA in pUC20. The library was transformed into competent E. coli XL-1 Blue cells. Colonies were screened by hybridization with the DIG-labelled probe.

One possible clone was detected, the cloned genomic fragment was digested with EcoRI and the subfragments were cloned into the EcoRI site of pUC20 for determination of the complete nucleotide sequence. During a three-step procedure the complete ORF of the putative GlcT, including the Shine-Dalgarno box, was cloned into the vector pT7-6 (Tabor & Richardson, 1985). The gene could be expressed under the control of the T7 promoter in E. coli BL21(DE3) (Stratagene). This construct was named pT7-6-glcT.

Expression and purification of GlcT. E. coli strain BL21(DE3) was transformed with pT7-6-glcT. The antiterminator protein GlcT was expressed by growing cells in TBY medium containing 37 °C until they had reached an A50 in of about 1. IPTG was subsequently added to a final concentration of 0.33 mM and cells were incubated for an additional 3 h at 37 °C.

Cells (4 g) were resuspended in 10 ml buffer A (Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM NaN3, 1 mM PMSF, 1 mM DTT) and disrupted by sonication. Cell debris was removed by centrifugation for 5 min at 30000 g and the supernatant was loaded on a Q-Sepharose column (3 x 10 ml; Pharmacia) equilibrated with buffer A. The proteins were eluted with a 0.6 M linear gradient of 0–6 M NaCl in buffer A. GlcT-containing fractions (0.5–0.9 M NaCl) were identified. The pool was adjusted to 25% ammonium sulfate and applied on
a Butyl TSK column (5 x 22 cm; Tosohas) pre-equilibrated with 25% ammonium sulfate in buffer A. The proteins were eluted with a 21 linear gradient (25-0% ammonium sulfate in buffer A). GlcT eluted at 2-0% ammonium sulfate; the pool was concentrated by ammonium sulfate precipitation (85%), the pellet was resuspended in 20 ml buffer A and loaded onto a Sephadex G75 gel filtration column (5 x 90 cm; Pharmacia) which was eluted with 21 buffer A. The GlcT pool was stored as an ammonium sulfate precipitate (85% ammonium sulfate). All purification steps were checked by SDS-PAGE (12% ; Laemmli, 1970).

**Phosphorylation of GlcT and separation of phosphorylated GlcT.** Phosphorylation was achieved by incubation of GlcT with enzyme I and HPn in a sixfold excess of GlcT. The reaction was performed in 25 mM Tris/HC1 buffer, pH 7.5, 2 mM MgC12, and 2-5 mM phosphoenolpyruvate. Phosphorylation was detected by using 10-12% native gels (Hjerten et al., 1965). For separation of phosphorylated GlcT, 3 nmol protein was used and after 30 min incubation at 37°C with 0.5 nmol enzyme 1/HPn the phosphorylation mixture was loaded for FPLC gel filtration (Superose 12; Pharmacia) at a flow rate of 0.5 ml min⁻¹.

**In vivo assay of GlcT activity.** In vivo assays were performed in E. coli DH5α (Sambrook et al., 1989) and B. subtilis QB5448 and GP109 (Stülke et al., 1997; Bachem & Stülke, 1998).

For studies in E. coli, the promoter region of glcA was translationally fused with gfp, encoding the green fluorescent protein. The promoter region was subcloned into the HimcII site of pUC20 through HaelIII restriction of pUC18-NB (Christiansen & Hengstenberg, 1996, 1999). Restriction with PstI led to cloning into the PstI site of pGFP (Clontech). The resulting construct was named pRAT/Tₖ−gfp. After cloning in pUC20 via EcoRI restriction, the insert was amplified by PCR using reverse and universal primers (Boehringer Mannheim). The fragment containing RAT/Tₖ−gfp was cloned into the Smal site located downstream of the glcT gene of pUC21-glcT (all amplified fragments were sequenced). For in vivo studies, the construct was used with RAT/Tₖ−gfp in the opposite orientation glcT (named pGG1). As a negative control a construct was used with the amplified fragment RAT/Tₖ−gfp cloned into the Smal site of pUC12 (named pGG0).

Cells of E. coli DH5α were transformed with pGG0 and pGG1. Fluorescence was detected after growth in TBY medium at 30 °C starting from an A₆₅₀ of 0.2. IPTG was added to a final concentration of 0.3 mM and growth was stopped at an A₆₅₀ of 1.0. The cells were centrifuged and resuspended in 0.8 ml 10 mM Tris/HC1, pH 7.5, 50 mM NaCl. Cells were disrupted by sonication, centrifuged at 10000 g for 1 h and the supernatant was used for fluorescence measurement with a spectrofluorometer (Jasco) at 395 nm excitation and 509 nm emission. As a measure of relative fluorescence, the fluorescence measurement was divided by the cell density.

For studies with B. subtilis, two constructs were made. (i) The glcT gene was cloned by KpnI/BamII restriction into the shuttle vector pHIT304 (Arantes & Lereclus, 1991) to give pHIT304–glcT. (ii) To clone the N-terminal RNA-binding domain (RBD), two primers were designed according to the amino acids 1-8 (pRBD1; 5′-AACCCGGGGCAAAAAGGCTGATTTAGCATTGAACTACGCTAG-3′) and 52-60 (pRBD2; 5′-ATATCTAGATATCTTCTTGGCTCT- AATTGAAAC-3′). Moreover, primer pRBD1 contains a B. subtilis Shine–Dalgarno box and a Smal site.

For PCR, 200 ng pUC21–glcT was used and the reaction was performed using standard procedures. The amplified fragment was cloned into the HimcII site of pUC21, sequenced and cloned into the vector pBQ200 through Smal restriction to give pBQ200-rbd.

**B. subtilis** was transformed with plasmids pHIT304–gfpT and pBQ200-rbd according to the two-step protocol described by Kunst & Rapoport (1995). Transformants were selected on SP plates containing chloramphenicol (5 µg ml⁻¹), kanamycin (5 µg ml⁻¹) or erythromycin plus lincomycin (1 and 25 µg ml⁻¹, respectively).

Quantitative studies of lacZ expression in B. subtilis in liquid medium were performed as follows. Cells were grown in CSE medium supplemented with the carbon sources indicated. Cells were harvested at an OD₆₀₀ of 0.6-0.8 for cultures in CSE medium and 0.8-1 for cultures in CSE medium with sugar. Cell extracts were obtained by treatment with lysozyme and DNase. β-Galactosidase activities were determined as previously described using ONPG as substrate (Miller, 1972). One unit is defined as the amount of enzyme which produces 1 nmol o-nitrophenol min⁻¹ at 28 °C.

**Liquid chromatography (LC)-MS analysis of non-phosphorylated and phosphorylated GlcT.** LC-MS/MS spectra were recorded on a Finnigan TQ 7000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. GlcT was dissolved in ammonium hydrogen carbonate buffer (pH 8.0), additionally purified through HPLC (C4 column; flow rate, 80 µl min⁻¹) and introduced on-line into the ESI source. Phosphorylated GlcT was rebuffed through G25 high trap columns (Pharmacia) in ammonium hydrogen carbonate buffer (pH 8.0) and prepared as described for non-phosphorylated GlcT.

For identification of the phosphorylation site, 2 nmol phosphorylated protein was digested with LysC protease (Promega) at 37 °C overnight. HPLC separation of the peptides was performed on a 300 µm × 25 cm C18 column at a flow rate of 4 µl min⁻¹. The peptides were eluted by a linear gradient (5-50%) of solvent B over 90 min (solvent A, 0.025% trifluoroacetic acid; solvent B, 0.02% trifluoroacetic acid, 80% acetonitrile).

**Limited LysC digestion.** For the digestion experiments, LysC protease (Promega) was used at a ratio of 1:60 (w/w, protease/substrate) as recommended by the supplier. For isolation of proteolytic fragments, the digestion reaction contained 4 nmol GlcT and 4 µg LysC protease and was incubated for 20 min at 37 °C. Purification was performed by FPLC gel filtration (Superose 12; Pharmacia). Fragments were identified by N-terminal sequencing with an Applied Biosystems Sequencer 473A.

**Assay of protein–RNA interaction by surface plasmon resonance analysis.** Sequence-specific protein–RNA interactions were detected by surface plasmon resonance analysis using the BIAcore X optical biosensor (Pharmacia Biosensor). 5'-Biotinylated synthetic RNAs were immobilized onto a streptavidin-containing chip. The RNA, dissolved in HBS-EP buffer, pH 7.5, was immobilized by immobilization and stimulation of the chip. The peptides were eluted by a linear gradient (5-50%) of solvent B over 90 min (solvent A, 0.025% trifluoroacetic acid; solvent B, 0.02% trifluoroacetic acid, 80% acetonitrile).

**Limited LysC digestion.** For the digestion experiments, LysC protease (Promega) was used at a ratio of 1:60 (w/w, protease/substrate) as recommended by the supplier. For isolation of proteolytic fragments, the digestion reaction contained 4 nmol GlcT and 4 µg LysC protease and was incubated for 20 min at 37 °C. Purification was performed by FPLC gel filtration (Superose 12; Pharmacia). Fragments were identified by N-terminal sequencing with an Applied Biosystems Sequencer 473A.

**Assay of protein–RNA interaction by surface plasmon resonance analysis.** Sequence-specific protein–RNA interactions were detected by surface plasmon resonance analysis using the BIAcore X optical biosensor (Pharmacia Biosensor). 5'-Biotinylated synthetic RNAs were immobilized onto a streptavidin-containing chip. The RNA, dissolved in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20), was coupled to the streptavidin surface of the sensor chip at a flow rate of 10 µl min⁻¹. Since 1000 resonance units (RU) might correspond to a surface concentration of 1 ng mm⁻², the different RNA molecules were immobilized to surface concentrations between 14 and 18 ng mm⁻². The standard running buffer was HBS-EP buffer and all reagents were introduced at a flow rate of 10 µl min⁻¹. GlcT from S. carnosus and B. subtilis was used at concentrations of about 1-6 µM. The sensor surface was regenerated between assays by injecting 20 µl 50 mM NaOH (in 1 M NaCl) to remove bound analyte. For processing the data, the program BIAevaluation 3.0 was used.
RESULTS

Cloning of the glcT gene from S. carnosus and overexpression and purification of GlcT

During cloning of glcA and glcB, encoding enzymes IICBA1 and IICBA2 (Christiansen & Hengstenberg, 1996, 1999), respectively, a partial ORF of 138 aa was identified upstream of the putative promoter of glcA. Sequence alignments indicated high similarity to the C-terminal part of members of the antiterminator proteins (Rutberg, 1997).

The corresponding nucleotide sequence was used to construct a DNA probe to screen a genomic library of S. carnosus. The complete ORF of the gene encoding GlcT was isolated as a fragment of 2258 bp. The fragment was subcloned into the vector pT7-6 and expressed in E. coli strain BL21(DE3).

Overexpression was analysed by SDS-PAGE (major band at 33 kDa). GlcT partially formed inclusion bodies. However, sufficient amounts of GlcT were detected in the supernatant. Purification was performed as described above and each step was analysed by SDS-PAGE. The mass of the purified protein was determined via ESI-MS (see Fig. 2). The measured mass of 33 504 Da confirmed the theoretical mass of 33 500 Da with the expected cleavage of the first amino acid, methionine, during expression in E. coli. From about 1 l culture we obtained a yield of about 11 mg purified protein. Interestingly, the purified protein showed a pattern of two bands after analysis on native PAGE (Fig. 1), suggesting a monomer–dimer distribution. On SDS-PAGE the pure protein was present as a single band around 30 000 Da with a trace of dimer at 60 000 Da.

In vivo assay of GlcT activity

Sequence alignments with the promoter region of the ptsG operon of B. subtilis (Stülke et al., 1997) revealed the existence of a RAT sequence in the promoter region of the glcA gene of S. carnosus. To identify whether the cloned gene indeed encodes an antiterminator protein, we investigated the biological activity concerning antitermination of glcA transcription. A translational fusion with the promoter region of glcA and the gene encoding green fluorescent protein as a reporter for possible transcription was constructed and expressed in E. coli (pGG1). In the presence of glcT and IPTG as inducer for GlcT synthesis, the relative fluorescence increased from 38 to 125 to 160 at final IPTG concentrations of 0, 0.02 and 0.2 mM, respectively, while the negative control (pGG0) showed no increase in fluorescence (relative fluorescence value about 5). These data show that the presence of GlcT enables constitutive expression of the glcA gene. Since the promoter region of glcB does not show an arrangement of a RAT/terminator sequence, we assume that the expression of glcB may be coupled partially to expression of glcA. Additional studies which were also performed in E. coli strains carrying deletions (ΔptsH crr and ΔptsG), encoding HPr/enzyme IIA<sup>Glc</sup> and enzyme IICB<sup>Glc</sup>, respectively) resulted in similar fluorescence intensities (data not shown). This leads to the assumption that the PTS of E. coli has no influence on the activity of S. carnosus GlcT.

Phosphorylation of GlcT by PTS components and its effect on the structure of GlcT

The enzyme I/HPr-dependent phosphorylation of B. subtilis GlcT at residue His104 has been suggested to cause inactivation of the protein (Bachem & Stülke, 1998). According to the proposed active dimeric state, phosphorylation was expected to lead to monomerization of GlcT. Phosphorylation of S. carnosus GlcT by the general components of the PTS was analysed by native PAGE and ESI-MS (see below). Two changes could be observed. First, on native gels the pattern changed upon phosphorylation. The band representing the monomeric protein disappeared. Second, the band of the dimer shifted. In general a band shift indicates an additional negative charge through a phosphoryl group. Consequently, this shift suggested a phosphorylation via the general components of the PTS (Fig. 1a). Surprisingly, in contrast to GlcT of B. subtilis, enzyme I/HPr-dependent phosphorylation of GlcT of S. carnosus clearly facilitated dimerization. The monomer–dimer distribution shifted almost quantitatively to the dimeric state. Separation via gel filtration confirmed this observation since two peaks were observed. Retention time of these two fractions suggests masses of 30 kDa (monomer) and 60 kDa (dimer). Phosphorylation caused partial disappearance of the monomer fraction (Fig. 1b).

According to genetic studies, the formation of the antiterminator/RAT complex should be carbohydrate-specific and dependent on complete enzyme IICBA1 as the sensor for glucose. Therefore, the influence of the glucose-specific IIBA domain on the monomer–dimer distribution was studied: neither the phosphorylated form of IIBA<sup>Glc</sup> nor the non-phosphorylated domains altered the oligomeric state of GlcT of S. carnosus. In addition, the interaction of spin-labelled IIBA in various phosphorylation states with GlcT was also investigated via electron paramagnetic resonance (EPR) spectrometry, but no interaction between GlcT and IIBA<sup>Glc</sup> could be detected. However, these data do rule out that intact glucose-specific enzyme IICBA of S. carnosus is still involved in regulation of GlcT under in vivo conditions.

Determination of the phosphorylation site

For several antiterminator proteins, multiple phosphorylation sites at conserved histidyl residues were observed (Stülke et al., 1998). Enzyme I/HPr-dependent phosphorylation of LiT leads to three phosphorylated residues, whereas in SacY two histidyl residues were phosphorylated (Lindner et al., 1999; Tortosa et al., 1997).

Since PTS-dependent phosphorylation of GlcT of S. carnosus has not been investigated via ESI-MS yet, the
number of phosphoryl groups per molecule GlcT was determined. In previous studies, phosphorylation was visualized by labelling with radioactive phosphate. This technique does not allow detection of the number of phosphates per molecule. A mass increase of 80 Da suggested a single phosphorylated residue (80 Da corresponds to one phosphoryl group; Fig. 2a). Since four highly conserved histidyl residues exist in the PRDs of antiterminator proteins, one of these four histidines might be the candidate for enzyme I/HPr-dependent phosphorylation. To localize the phosphorylated residue, phosphorylated GlcT was digested with the endoproteinase LysC and the masses of the generated fragments were measured by ESI-MS.

All observed fragments corresponded to the predicted masses except a single peptide with a mass of 1829.2 Da. This mass shows good agreement to the mass of the fragment containing His105 with an additional phosphoryl group (Fig. 2b). Three lines of evidence suggest that His105 is phosphorylated. (i) Acid-lability of the phosphorylated GlcT pointed to a phosphorylated histidine residue (data not shown). (ii) Residue His104 was described as the target for phosphorylation of the closely related GlcT of *B. subtilis* (Bachem & Stülke, 1998). (iii) Residue His105 is the only histidine present in the putatively phosphorylated fragment.

Considering the *in vivo* experiments, phosphorylation studies with HPr of *E. coli* and *B. subtilis* were performed. As expected because of the high similarities, GlcT was efficiently phosphorylated by *B. subtilis* HPr and this phosphorylation led to dimerization. In contrast, HPr from *E. coli* is not able to phosphorylate GlcT to a detectable extent.

**In vivo assays of GlcT of *S. carnosus* in *B. subtilis***

As already mentioned, the two *ptsG-RAT* sequences of *B. subtilis* and *S. carnosus* are highly similar (Langbein *et al.*, 1999). Since the possible complementation of these two systems was expected, *B. subtilis* strains were transformed first with plasmids containing the whole *glcT* gene (pHT304-*glcT*) and with the sequence en-
Table 1. Effects of *S. carnosus* GlcT and N-terminal domain on the expression of the *ptsGHI* operon in *B. subtilis*

Both strains contain a ∆LA *ptsG*–*lacZ* translation fusion. β-Galactosidase activity is expressed in units (mg protein)$^{-1}$.

<table>
<thead>
<tr>
<th><em>B. subtilis</em> strain (construct)</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSE (no glucose)</td>
</tr>
<tr>
<td>(a) GlcT</td>
<td></td>
</tr>
<tr>
<td>QB5448(pHT304)</td>
<td>11</td>
</tr>
<tr>
<td>GP109(pHT304)</td>
<td>4</td>
</tr>
<tr>
<td>QB5448(pHT304-glcT)</td>
<td>1438</td>
</tr>
<tr>
<td>GP109(pHT304-glcT)</td>
<td>1204</td>
</tr>
<tr>
<td>(b) N-terminal domain</td>
<td></td>
</tr>
<tr>
<td>QB5448(pBQ200)</td>
<td>11</td>
</tr>
<tr>
<td>GP109(pBQ200)</td>
<td>3</td>
</tr>
<tr>
<td>QB5448(pBQ200-rbd)</td>
<td>1115</td>
</tr>
<tr>
<td>GP109(pBQ200-rbd)</td>
<td>1310</td>
</tr>
</tbody>
</table>

coding the first 60 aa of GlcT (pBQ200-rbd). Two strains (wild-type QB5448 and ΔglcT mutant GP109) with an insertion in the *amyE* locus, including the *lacZ* gene fused with the *ptsG*-promoter, were used. This fusion allows convenient GlcT activity tests via the expression of β-galactosidase (Stülke *et al.*, 1997; Bachem & Stülke, 1998). As shown in Table 1(a), GlcT of *S. carnosus* recognizes the *ptsG*-RAT sequence of *B. subtilis* and causes antitermination, as visualized by β-galactosidase activity in both strains. GlcT of *S. carnosus* was active with or without glucose added to the growth medium. The first 60 aa forming the RBD also showed this constitutive antitermination activity (Table 1b).

### Interaction studies by surface plasmon resonance

Since antitermination in *B. subtilis* by *S. carnosus* GlcT also occurred *in vivo* we studied the potential of GlcT for RAT binding *in vitro* via surface plasmon resonance. Langbein *et al.* (1999) used the BIAcore technique for measurement of specific interactions between the RBD of *B. subtilis* GlcT and the RAT sequence. The data with *S. carnosus* GlcT confirmed that the dimer is the active form because of its higher affinity to RAT (Fig. 3). Since the same affinity was observed for the non-phosphorylated dimer (isolated via gel filtration from purified protein), the antitermination in *E. coli* using the green fluorescent protein construct could be explained by spontaneous dimerization due to the high concentration of the overproduced protein. Dimerization via phosphorylation can be excluded: *E. coli* HPr was unable to phosphorylate GlcT.
Staphylococcus carnosus PTS regulation

**Fig. 3.** Comparison of the affinity of GlcT to RAT analysed by the optical BIAcore technique. Resonance units (RU) are a relative measure of the amount of antiterminator bound to the RAT RNA attached to the surface of a sensor chip. In addition, the time-dependence of complex formation is monitored. For the assays a protein concentration of about 1 µM (monomeric GlcT; dashed line) and 0.5 µM (dimeric, non-phosphorylated GlcT, solid/dotted line, respectively) was used.

**Fig. 4.** Affinity of *B. subtilis* GlcT (solid line) and *S. carnosus* GlcT (dashed line) to the ptsG-RAT of *B. subtilis* analysed by the BIAcore technique. In spite of the high similarity between the two ptsG-RAT sequences of *S. carnosus* and *B. subtilis*, there is still a significant difference in affinity between these two antiterminator proteins (Fig. 4). It is plausible that the exchange of three nucleotides in RAT together with differences in the protein primary structure influence the affinity of GlcT to RAT.

**LysC digestion experiments**

Limited proteolysis experiments give an insight into the domain organization of a protein. Cleavage sites of proteases are protected through the compact structure of a domain. Partial proteolysis experiments with the dimeric and monomeric form of the protein showed the same digestion pattern in SDS-PAGE. After about 60 s, the digestion generated a fragment with a size in a range of 26–27 kDa. This fragment was resistant to further cleavage for about 30 min. Later, two other fragments with a size in a range of 13–16 kDa appeared. To identify the first fragment it was isolated via gel filtration and analysed by N-terminal sequencing. The fragment started with amino acid Ala66 and had a molecular mass of 26099 Da which pointed to a stable C-terminal part of the protein.

Unexpectedly, phosphorylation by the general components of the PTS is still possible as is dimerization (Fig. 5).

**DISCUSSION**

**Role of HPr-dependent phosphorylation of His105 in GlcT of *S. carnosus***

Compared to the phosphorylation pattern of other antiterminators of the bgl-sac family which show multiple phosphorylation of GlcT, *S. carnosus* is phosphorylated only once at His105. A more detailed
inspection of the ESI mass spectrum reveals another very minor peak indicative of a second phosphorylation. Phosphorylation at His105 enhances dimer formation. There is also a dimer present in the non-phosphorylated state which was shown to bind to the B. subtilis RAT sequence with a similar affinity as the phosphodimer. The spontaneous dimer is probably generated due to the high concentrations of the antiterminator protein during overexpression as described for LicT (Krüger & Hecker, 1995). Under physiological conditions, as they occur during induction of glucose metabolism in the wild-type cell, antiterminator protein will be present at a very low concentration, according to Northern hybridization experiments (indicative of the amount of mRNA: J. Stülke, personal communication) and will even be diluted by up to 3000 different proteins of the bacterial cytoplasm. Thus HPr-dependent phosphorylation may be essential for formation of the dimer which performs antitermination as confirmed by surface plasmon resonance experiments.

Since His105 is a conserved histidine residue in PRD-I of GlcT, this result seems to be contradictory to the conception of PRD-I as a target of negative regulation of antiterminator proteins (Stülke et al., 1998). We suggest that HPr-dependent phosphorylation of GlcT followed by dimer formation may be a control device to ensure the proper function of the general PTS components enzyme I and HPr. Induction of glucose-specific enzyme II only is reasonable if intact general PTS components are available for enzyme II phosphorylation.

On the other hand HPr is involved in regulation of carbohydrate metabolism in Gram-positive bacteria through ATP-dependent phosphorylation at Ser46 (Kravanja et al., 1999). This regulatory phosphorylation of HPr inhibits phosphoenolpyruvate-dependent phosphoryl transfer to HPr and thus would also affect GlcT activity. This might be a control mechanism to inhibit excessive induction of glucose-specific enzyme II.

**Dimerization potential of structural domains of S. carnosus GlcT**

As shown in B. subtilis glucose metabolism, the N-terminal domain of GlcT interacts as a dimer with the corresponding RAT sequence. This leads to constitutive synthesis of glucose-specific PTS (Bachem & Stülke, 1998). Structural studies on the similar RBD of the SacY antiterminator protein suggested a dimer as the RNA binding form (van Tilbeurgh et al., 1997; Manival et al., 1997).

To further estimate whether isolated PRDs without the RBD possess dimerization potential, PRD-I and PRD-II were produced by treatment with the endoproteinase LysC. This fragment could still be phosphorylated by the general PTS components, confirming the definition of the functional independence of domains. Moreover, this fragment still dimerizes. The in vivo assays in B. subtilis with the RBD from GlcT of S. carnosus suggested dimerization because of the observed antitermination. These results led to the conclusion that two dimerization motifs exist in a single molecule. One motif is localized in the RBD causing spontaneous dimerization. This process may be responsible for the monomer–dimer pattern which was observed for the overexpressed, non-phosphorylated GlcT. The second motif is located in the PRDs, as shown for the E. coli antiterminator protein BglG (Boss et al., 1999). Dimerization is under control of the phosphorylation via the general PTS components.

**Possible mechanism for glucose-specific antitermination**

In the absence of glucose the phosphodimer must be inactivated somehow or be unavailable for antitermination. Upon the addition of glucose as an inducer, the phosphorylated GlcT should become accessible and interact with the RAT sequence. So far we have not been able to detect any effect on the monomer–dimer distribution of phosphorylated GlcT by the isolated domains IIBA or IA of the glucose-specific enzyme IICBA. No additional phosphorylation in stoichiometric amounts of the potential histidyl residues requiring enzymes IA, IIB or IIA could be detected in vitro with mass spectrometric methods. In vivo studies with GlcT of B. subtilis showed that a mutant of IIBA (H620A) which could no longer be phosphorylated but which contained an intact IIC domain still showed glucose-inducible antitermination (Bachem & Stülke, 1998). Thus an additional direct phosphorylation or dephosphorylation of phosphorylated GlcT by enzyme IIBA alone could not be observed in vitro and may be not involved in glucose induction. However, there is still the possibility that the IIC domain may be required for an additional phosphorylation/dephosphorylation of phosphorylated GlcT.

During in vivo studies with B. subtilis GlcT, S. carnosus GlcT also led to antitermination, as monitored by the lacZ fusion, but induction of antitermination could not be initiated by adding glucose. GlcT of S. carnosus behaved constitutively, similar to the N-terminal fragment of B. subtilis GlcT which is dimeric, as observed for the homologous fragment of the SacY antiterminator but the specific interaction with glucose-specific enzyme II probably mediated by the PRDs is lacking. The studies described above confirm that the antiterminator proteins of the bgl-sac family perform antitermination in the dimeric state. For S. carnosus GlcT phosphorylation of His105 clearly enhances dimer formation. The basal level dimeric enzyme IICBA – probably in its phosphoform during starvation – must somehow complex the dimeric GlcT which prevents binding to RAT. The binding of glucose to the phosphorylated enzyme IICBA should then release the GlcT dimer now capable of performing antitermination. In the in vivo studies with S. carnosus GlcT, the complexing and releasing procedure inside the B. subtilis cell may not function properly due to imperfect protein interaction between B. subtilis enzyme IICBA and S. carnosus GlcT. An
alternative explanation would be an excessive expression of the *S. carnosus* GlcT protein in *B. subtilis*. The amount of free unbound GlcT may then also lead to constitutive antitermination.

Preliminary support for this tentative mechanism of antiterminator release may be the observation that membrane fragments of *S. carnosus* containing induced enzyme IICBA appeared to specifically remove dimeric GlcT from the monomer–dimer mixture. Such a mechanism would require a strong conformational change in the enzyme IICBA structure upon binding and phosphorylation of the carbohydrate. Such an effect has been observed with the mannitol-specific enzyme II during micro-calorimetric experiments (Meijberg et al., 1998). According to these studies on the mannitol-specific enzyme II (also of the IICBA type) this protein was shown to be active as a dimer (Pas et al., 1988). In the case of the mannitol-specific enzyme II a very strong, unexpected conformational change of the IIBA domains upon binding of the substrate or its analogue was observed. Such a massive conformational change could lead to the release of the dimeric antiterminator complexed by enzyme IICBA. Further biochemical and genetic experiments will be necessary to support or abandon the proposed glucose-dependent induction mechanism.

**ACKNOWLEDGEMENTS**

The authors would like to thank Fritz Titgemeyer for providing the *E. coli* FT1 strain and Dr Robillard for providing HPr from *E. coli*. This research was supported by SFB394 of the Deutsche Forschungsgemeinschaft.

**REFERENCES**


Schnetz, K. & Rak, B. (1990). Beta-glucoside permease represses the bgI operon of Escherichia coli by phosphorylation of the antiterminator protein and also interacts with glucose-specific enzyme III, the key element in catabolite control. Proc Natl Acad Sci USA 87, 5074–5078.


Received 8 March 2000; revised 24 May 2000; accepted 23 June 2000.