Staphylococcal phosphoenolpyruvate-dependent phosphotransferase system – two highly similar glucose permeases in *Staphylococcus carnosus* with different glucoside specificity: protein engineering *in vivo*?

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Previous sequence analysis of the glucose-specific PTS gene locus from *Staphylococcus carnosus* revealed the unexpected finding of two adjacent, highly similar ORFs, *glcA* and *glcB*, each encoding a glucose-specific membrane permease EIICBA^{Glc}. *glcA* and *glcB* show 73 % identity at the nucleotide level and *glcB* is located 131 bp downstream from *glcA*. Each of the genes is flanked by putative regulatory elements such as a termination stem-loop, promoter and ribosome-binding site, suggesting independent regulation. The finding of putative *cis*-active operator sequences, CRE (catabolite-responsive elements) suggests additional regulation by carbon catabolite repression. As described previously by the authors, both genes can be expressed in *Escherichia coli* under control of their own promoters. Two putative promoters are located upstream of *glcA*, and both were found to initiate transcription in *E. coli*. Although the two permeases EIICBA^{Glc1} and EIICBA^{Glc2} show 69 % identity at the protein level, and despite the common primary substrate glucose, they have different specificities towards glucosides as substrate. EIICBA^{Glc1} phosphorylates glucose in a PEP-dependent reaction with a K_m of 12 μ M; the reaction can be inhibited by 2-deoxyglucose and methyl β -D-glucoside. EIICBA^{Glc2} phosphorylates glucose with a K_m of 19 μ M and this reaction is inhibited by methyl α -D-glucoside, methyl β -D-glucoside, *p*-nitrophenyl α -D-glucoside, *o*-nitrophenyl β -D-glucoside and salicin, but unlike other glucose permeases, including EIICBA^{Glc1}, not by 2-deoxyglucose. Natural mono- or disaccharides, such as mannose or *N*-acetylglucosamine, that are transported by other glucose transporters are not phosphorylated by either EIICBA^{Glc1} nor EIICBA^{Glc2}, indicating a high specificity for glucose. Together, these findings support the suggestion of evolutionary development of different members of a protein family, by gene duplication and subsequent differentiation. C-terminal fusion of a histidine hexapeptide to both gene products did not affect the activity of the enzymes and allowed their purification by Ni²⁺-NTA affinity chromatography after expression in a *ptsG* (EIICB^{Glc}) deletion mutant of *E. coli*. Upstream of *glcA*, the 3' end of a further ORF encoding 138 amino acid residues of a putative antiterminator of the BglG family was found, as well as a putative target DNA sequence (RAT), which indicates a further regulation by glucose specific antitermination.

Keywords: glucose-specific phosphotransferase system, *Staphylococcus carnosus*, membrane protein, regulation, kinetics

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Abbreviations: CRE, catabolite-responsive element; NTA, nitrilotriacetic acid; PEP, phosphoenolpyruvate; PTS, phosphotransferase system.

INTRODUCTION

The phosphotransferase system (PTS) catalyses transport and phosphorylation of carbohydrates in various obligate and facultative anaerobic bacteria. This system has also been implicated in chemotaxis and in regulation of numerous catabolic pathways (Erni, 1992; Hengstenberg *et al.*, 1993; Postma *et al.*, 1993; Lengeler *et al.*, 1994). Enzyme I (EI) and the histidine-containing phosphocarrier protein (HPr) are soluble cytoplasmic proteins that are involved in the transport of all PTS sugars and are thus designated as general proteins of the PTS. During the transfer of the phosphoryl group from phosphoenolpyruvate (PEP) to the substrate, both proteins are phosphorylated on a histidyl residue. Enzymes II (EII) are sugar-specific permeases which consist of three domains (EIIA, EIIB, EIIC). IIA and IIB are hydrophilic and cytoplasmic, and both contain a phosphorylation site, which is a histidyl residue (IIA) and a cysteal residue (IIB), respectively. Exceptions are described for IIB^{Man} from *Escherichia coli*, IIB^{Sor} from *Klebsiella pneumoniae* and IIB^{Fru} from *Bacillus subtilis*, which are phosphorylated at a histidyl residue (Erni *et al.*, 1989; Wöhrl & Lengeler, 1990; Martin-Verstraete *et al.*, 1990). IIC is a transmembrane domain and serves as the binding site and translocator for the substrate. Depending on the PTS and the bacterial species, the domains are expressed independently or as fusion proteins, in which the domain organization may vary (Saier & Reizer, 1994).

Recently, we reported the cloning and sequencing of two EII-coding genes, *glcA* and *glcB*, from *Staphylococcus carnosus* and their expression in *E. coli* (Christiansen & Hengstenberg, 1996). Both genes showed high similarities to members of the glucose-specific subfamily of PTS permeases. Despite their close proximity, sequence analysis indicated an independent individual regulation of gene expression for *glcA* and *glcB*.

The three EII domains are fused in both permeases (EIICBA). Each protein is physiologically active in *E. coli* WA2127Δ*ptsG*::Cm^r and was shown to restore glucose fermentation in the *E. coli* mutant. Since EII permeases show overlapping specificity, we addressed the question whether these two glucose transporters have different substrates. To study the regulation of glucose uptake and metabolism in the Gram-positive bacterium *S. carnosus*, an important organism in food production, a further characterization of the two highly similar permeases is necessary. Here, we describe the purification of the two proteins EIICBA^{Glc1} and EIICBA^{Glc2} to apparent homogeneity, and the characterization of their substrate specificities in an *in vitro* fluorimetric assay. Additionally, the potential to initiate transcription of the two promoters preceding *glcA* was analysed by deletion studies.

METHODS

Chemicals. Restriction enzymes, T4-DNA ligase, DNase and RNase were obtained from Bethesda Research Laboratories; Triton X-100, glucose-6-phosphate dehydrogenase,

Klenow fragment of polymerase I, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, ampicillin and chloramphenicol were obtained from Boehringer Mannheim; Bacto-Tryptone, yeast extract and MacConkey agar base were from Difco. The Ni²⁺-nitrilotriacetic acid (NTA) expression and purification kit was from Qiagen. PVDF membranes were from Bio-Rad. Other reagents were commercial products of the highest purity available.

Bacterial strains, plasmids and growth conditions. *E. coli* TG1 (Sambrook *et al.*, 1989) was used for cloning experiments. Expression of the EIICBA^{Glc} proteins was performed in *E. coli* WA2127Δ*ptsG*::Cm^r (Δ*ptsG ptsLPM* Cm^r *his leu met lac supE*[±] r_k[−] m_k[−]) (Buhr *et al.*, 1994), obtained from B. Erni, Bern. *S. carnosus* TM300 (Schleifer & Fischer, 1982) was obtained from F. Götz, Tübingen. pUC18/19 (Vieira & Messing, 1985) were used for cloning experiments. LB medium and growth conditions were as described previously (Christiansen & Hengstenberg, 1996). Fusion of histidine hexapeptide to the gene products was achieved by using the pQE vectors from Qiagen.

Preparation of cell membranes. Cells of *E. coli* WA2127Δ*ptsG*::Cm^r were grown overnight in 2 l LB medium and harvested by centrifugation. The cell paste (approx. 10 g) was resuspended in 2 vols standard buffer Sb (0.05 M Tris/HCl, pH 7.5, 10^{−4} M DTT, 10^{−4} M PMSF, 10^{−4} M Na₃N₃, 10^{−4} M EDTA). The cells were disrupted by sonication with a Branson sonifier B12. The crude extract was centrifuged for 15 min at 10000 g and 4 °C. Membranes were collected at 170000 g for 4 h (4 °C), resuspended in 20 vols Sb and sedimented under the same conditions to remove remaining cytoplasmic contaminants. The washed membrane fractions were suspended in the required volume of Sb.

In vitro EII^{Glc} activity test and inhibitor kinetics. The PTS-dependent phosphorylation of glucose was measured by a coupled reaction of glucose-6-phosphate dehydrogenase in 250 μl samples at 37 °C, containing 6 mM MgCl₂, 0.5 mM NADP⁺, 1 mM PEP, 5 μg EI from *S. carnosus* (80 pmol), 5 μg HPr from *S. carnosus* (530 pmol), glucose-6-phosphate dehydrogenase (0.25 units), 10 μl membrane resuspension (1 mg membrane fraction is resuspended in 10 μl Sb), and varying amounts of glucose (3.125–100 nmol). The reaction was started by adding HPr. Increments of NADPH concentration were detected in an Eppendorf fluorimeter 1030 (primary filter 313–366 nm, secondary filter 400–3000 nm).

The specificity of the EII^{Glc} was tested by inhibition of the reaction by potential substrate analogues. A negative control was performed by adding the inhibitors as substrates.

The reaction was performed using a glucose concentration five times higher than the calculated *K_m* value, and a 10-, 100- or 1000-fold excess of the potential inhibitor was added. In the case of an inhibition, glucose-specific Michaelis–Menten kinetics were repeated in the presence of different concentrations of inhibitor (1-, 2-, 5- and 100-fold the *K_i* value) and the inhibition constants were calculated by nonlinear curve-fitting. In the case of a quenching effect by the inhibitor on the detection of fluorescence (*o*- and *p*-nitrophenyl glucosides), the concentration-dependent factor was determined and taken into account.

Construction of plasmids. For expression of His-tagged EII^{Glc1} and EII^{Glc2}, the corresponding genes were cloned into the *Bam*HI restriction site of pQE, upstream of the histidine-hexapeptide coding region. Therefore, PCR was performed using the pUC universal primer and the oligonucleotides 3'-ATCAATTTAACCCTAGGGGTA-5' (*glcA*) or 3'-CCACTT-

ATTACCTAGGACAA-5' (*glcB*) (annealing temp. 42 °C) to introduce a *Bam*HI restriction site (underlined bases) with concomitant removal of the termination codons of the genes. A 192 bp *Eco*RV-*Bam*HI 3' fragment (*glcA*) and a 157 bp *Hinc*II-*Bam*HI 3' fragment (*glcB*) of the corresponding PCR products were isolated, sequenced and used to substitute the corresponding sequence in *glcA* and *glcB*, respectively. The modified genes were ligated into pQE plasmids, so the gene products were extended by the amino acids LDRS(H)₆ (EII^{Glc}1) or GS(H)₆ (EII^{Glc}2), respectively.

Two potential promoters P₀ and P₁ are located upstream of *glcA*. P₁ is flanked by the two restriction sites *Hin*FI and *Mn*II, which facilitated its removal (*glcA*-ΔP₁). *Mn*II was used to delete both promoters (*glcA*-ΔP₀₁), whereas P₀ was deleted by unidirectional deletion of DNA by exonuclease III and mung bean nuclease (*glcA*-ΔP₀).

Purification of EIICBA^{Glc}1 and EIICBA^{Glc}2. Both EII^{Glc} permeases were purified by using the Ni²⁺-NTA metal chelate system according to the procedure of the manufacturer. To improve the expression of the fusion proteins, the coding sequences were recloned into pUC18.

Alkaline wash of membranes. Membrane fractions (1 g) of *E. coli* WA2127Δ*ptsG*::Cm^r, expressing the desired gene product of *glcA* or *glcB*, were resuspended in 10 vols alkaline buffer [0.05 M Tris/HCl, pH 8.0, 10⁻⁴ M PMSF, 10% (v/v) glycerol, 10 mM glucose, 5 mM 2-mercaptoethanol]. The pH was adjusted to 12 (EII^{Glc}1-His) and 11.5 (EII^{Glc}2-His), respectively. After incubation for 15 min at room temperature, the pellet was harvested by centrifugation at 170 000 g, 4 °C, for 3.5 h.

Solubilization of membrane proteins. The membrane fraction after the alkaline wash (approx. 300 μg) was resuspended in 10 vols solubilization buffer (0.05 M Tris/HCl, pH 7.5, 10⁻⁴ M PMSF, 10⁻⁴ M Na₂S₂O₃, 5 mM 2-mercaptoethanol). Triton X-100 (final concn 1%, w/v) and NaCl (final concn 100 mM) were added and the proteins solubilized for 1 h with continuous stirring at 4 °C. After centrifugation at 170 000 g for 1 h, the supernatant was suitable for Ni²⁺-NTA affinity chromatography.

Ni²⁺-NTA affinity chromatography. The supernatant containing the solubilized proteins was applied to a column containing 0.5 ml Ni²⁺-NTA suspension, equilibrated with 20 vols solubilization buffer containing 1% Triton X-100. After washing with 20 vols solubilization buffer + 0.8% Triton X-100 and 6 vols solubilization buffer + 0.8% Triton X-100 and 25 mM imidazole, EII^{Glc} proteins were eluted with 4 vols elution buffer (solubilization buffer + 0.8% Triton X-100, 100 mM imidazole).

Ion-exchange chromatography. In case of residual contaminating proteins, an ion-exchange chromatography step was included. The eluted fractions of the Ni²⁺-NTA column were supplied to a column containing 1 ml Fractogel EMD DMAE 650 (S), equilibrated with 10 vols solubilization buffer + 0.8% Triton X-100. After washing with 10 vols of the same buffer, proteins were eluted with a linear gradient of NaCl (0–0.4 M, 5 ml).

PAGE. SDS-polyacrylamide gels were prepared according to Laemmli (1970). Protein concentration was determined according to the method of Peterson (1977).

Blotting to PVDF membranes. Protein transfer from SDS-PAGE gels to PVDF membranes was carried out in a Biometra 'Semi-dry Fast Blot'. The transfer buffer contained 0.09 M Tris/borate, pH 8.0, 1 mM EDTA, 20% methanol, 0.05% SDS. Gels were stained in 50% methanol, 0.1% Coomassie R250, and destained in 50% methanol.

N-terminal sequencing of proteins. Purified proteins EIICBA1^{Glc}-His and EIICBA2^{Glc}-His (approx. 20 μg) were blotted to

PVDF membranes and, after staining, protein-containing membrane slices were cut out and sequenced on a gas-phase sequencer according to Hewick *et al.* (1981).

RESULTS AND DISCUSSION

Expression and purification of EIICBA^{Glc}1-His and EIICBA^{Glc}2-His

In this report we describe for the first time the overexpression, purification and functional analysis of two glucose-specific EIICBA PTS permeases. It has already been established that *glcA* and *glcB* complement a *ptsG* mutant of *E. coli*. The *E. coli* WA2127Δ*ptsG*::Cm^r strain used for complementation lacks all EII^{Glc} and has a defect in the mannose-PTS, resulting in a glucose-nonfermenting phenotype. Cells transformed with pUC18 containing *glcA* or *glcB* formed red colonies on MacConkey agar containing glucose.

To enable a fusion of the histidine hexapeptide coding region of the pQE plasmids to the 3' end of *glcA* or *glcB*, the stop codon was removed and the genes were cloned into pQE as described in Methods. After expression using the pQE system, both proteins (EII^{Glc}1-His and EII^{Glc}2-His) complemented the *ptsG* deletion mutant *E. coli* WA2127Δ*ptsG*::Cm^r. Membrane fractions were collected and shown to phosphorylate glucose in the PEP-dependent reaction of the *in vitro* assay, although 15 times more slowly than after expression via the pUC system (see Fig. 2; data shown for pQE17-*glcA*.)

To increase the level of expression, the modified genes (*glcA*-his and *glcB*-his) were cloned into pUC18 plasmids in the opposite orientation to the *lac* promoter. Expression of these plasmids (pUC18-*glcA*-his and pUC18-*glcB*-his) restored the glucose fermentation of *E. coli* WA2127Δ*ptsG*::Cm^r and membrane fractions of those transformed cells showed a V_{max} of glucose phosphorylation identical to the wild-type proteins expressed via the pUC system. Moreover, the reaction of the His-tagged proteins gave the same K_m values in the *in vitro* activity test compared to the corresponding gene products without His-tag, showing that the C-terminal extension does not influence the functionality of the EII^{Glc}.

Alkaline washing of the isolated membranes at pH 12.0/pH 11.5 retained 15.5%/17.4% of the proteins and 74%/78% of the EII^{Glc} activity in the case of EII^{Glc}1-His/EII^{Glc}2-His. Approximately 55% of the membrane-bound EII^{Glc} activity could then be solubilized with Triton X-100. Varying amounts of Triton X-100 and NaCl did not improve the yield of solubilized activity, whereas other detergents were even less efficient. After adsorption to Ni²⁺-NTA and washing with buffer containing 25 mM imidazole, 50–60% of the total activity could be eluted with 100 mM imidazole. Residual contamination with proteins of a lower molecular mass (Fig. 1b, lane 5) was eliminated by ion-exchange chromatography on Fractogel EMD DEAE 650 (S). The yield of this additional purification step was 95%. The data are summarized in Table 1; Fig. 1 shows the purity reached in each of the chromatography steps. The

Fig. 1. Fractions of the purification steps from EIICBA^{Glc1} (a) and EIICBA^{Glc2} (b) separated by SDS-PAGE (10%). Lane 1, 75 µg membrane protein; lane 2, 75 µg membrane protein after alkaline treatment; lane 3, 30 µl supernatant after solubilization with Triton X-100 (40 µg protein); lane 4, 30 µl flowthrough of the Ni²⁺-NTA column (40 µg protein); lane 5, 3 µg protein eluted from the Ni²⁺-NTA column; lane 6 (in a), 3 µg protein eluted from the Ni²⁺-NTA column, heated to 80 °C for 5 min; lane 6 (in b), 3 µg protein eluted from the Fractogel EMD DEAE 650 (S) column; lane 7, 3 µg protein eluted from the Fractogel column, heated to 80 °C for 5 min. ST, size standards.

identity of the purified proteins was verified by 10 cycles of Edman degradation.

SDS-PAGE analysis of the purified proteins revealed

four tightly spaced bands in the case of EIICBA^{Glc1} and two bands in the case of EIICBA^{Glc2}, which in both cases were not separable by further chromatography but could be coalesced into a single band by heating to 80 °C for 5 min (Fig. 1a, lane 6; Fig. 1b, lane 7). This can be explained by different SDS binding properties after thermal breakdown of folded protein structures (Heller, 1978). In the case of EIICBA^{Glc1}, heating resulted in partial aggregation of protein molecules. The apparent molecular masses of the purified proteins, as revealed by SDS-PAGE, are lower than the calculated molecular masses of the His-tagged proteins; this can be explained by an increased binding of SDS to the strongly hydrophobic C-domains of these proteins. These unusual electrophoretic mobilities of proteins in SDS-PAGE are known as heat-modifiability and have been observed for many other membrane proteins, such as FhuA, FhuB, LacY, SecY, RodA, MalG and OmpA (Köster & Braun, 1986; Ried *et al.*, 1994; Locher & Rosenbusch, 1997) and even for other EII proteins (Lee & Saier, 1983; Bramley & Kornberg, 1987; Peters *et al.*, 1995).

That similar proteins show either an increased or decreased electrophoretic mobility after heating prior to SDS-PAGE has been shown for modified variants of the OmpA protein. Whereas the wild-type protein has a lower mobility, the R236V variant lacks modifiability and the membrane-spanning N-terminus (OmpA171) has an increased mobility after heat treatment (Ried *et al.*, 1994). In contrast, a circular permutation (2341) of the OmpA N-terminus has a decreased mobility under the same conditions (Koebnik & Krämer, 1995).

With the purification method described, we were able to isolate 200 µg (EIICBA^{Glc1}) or 160 µg (EIICBA^{Glc2}) purified protein from 10 g wet cell paste. A large-scale purification would provide enough purified proteins for production of antibodies and for crystallization of the proteins to study expression, structure and function. Crystallization seems to be more likely for EIICBA fusion proteins compared to EIICB molecules because of the higher proportion of hydrophilic amino acids. Structure analysis would give insight into function of the widely distributed bacterial PTS transporters which are also involved in bacterial signal transduction.

Table 1. Purification of EIICBA^{Glc1}-His and EIICBA^{Glc2}-His

One unit (U) is the amount of enzyme that catalyses the phosphorylation of 1 nmol glucose min⁻¹. In the case of EIICBA^{Glc1}-His, the Fractogel chromatography was not performed.

Purification step	EIICBA ^{Glc1}					EIICBA ^{Glc2}				
	Total protein [mg (g membranes) ⁻¹]	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield (%)	Total protein [mg (g membranes) ⁻¹]	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Membrane	250	5900	23.6	1	100	250	2050	8.2	1	100
Membrane, alkali treated	38.7	4373	113	4.8	74	43.6	1591	36.5	4.5	78
Solubilization	8.5	2499	294	12.5	40	13.5	850	62.9	7.7	42
Ni ²⁺ -NTA chromatography	0.2	1204	6019	255	20.4	0.2	529	2644	322	26
Fractogel chromatography	—	—	—	—	—	0.16	500	3125	381	24

Fig. 2. Michaelis-Menten kinetics of glucose phosphorylation by different preparations of EII^{Glc}. The reaction was measured by a coupled reaction as described in Methods, using as EII^{Glc} 10 μ l membrane resuspensions (1 mg membrane + 10 μ l buffer) from *E. coli* after expression of the different plasmid constructions indicated. pUC18-*glcA* and pUC18-*glcB*, EII^{Glc1} and EII^{Glc2}, respectively, after expression of the genes in pUC; pUC18-*glcA*, TX and pUC18-*glcB*, TX, the same membranes after adding Triton X-100 for solubilization (the same reactions are carried out by 1.2 μ g purified and solubilized EII^{Glc1} and 0.8 μ g purified and solubilized EII^{Glc2}, respectively). ΔP_0 , ΔP_1 , ΔP_{01} , EII^{Glc1} after expression of the different *glcA* promoter deletion constructs in pUC18. pQE17-*glcA*, EII^{Glc1} after expression of the gene in pQE. WT, wild-type membranes. The calculated K_m values for EII^{Glc1} and EII^{Glc2} are 12 μ M and 19 μ M for membrane preparations; after solubilization the values are 30.5 μ M and 37.2 μ M, respectively.

In vitro test of EII^{Glc} activity and kinetics of inhibitors

After expression of pUC18-*glcA* and pUC18-*glcB* in *E. coli* WA2127 Δ ptsG::Cm^r, isolated membranes had, respectively, a 45-fold and 15-fold increased rate of phosphorylation of glucose compared to equal amounts of cell membranes from *S. carnosus* (Fig. 2). The K_m values of the glucose phosphorylation were 12 μ M and 19 μ M for cell membranes after expression of pUC18-*glcA* and pUC18-*glcB*, respectively, similar to the K_m value obtained for the same reaction performed by use of membrane fractions isolated from *S. carnosus* (approx. 5 μ M; data not shown). The addition of the His-tag did not alter the kinetic properties. However, incubation with Triton X-100 led to slightly increased K_m values of PEP-dependent glucose phosphorylation by the products of *glcA* and *glcB*, whether membrane-bound or purified. Closer analysis of the Triton X-100-dependent kinetics of the EII proteins showed that glucose phosphorylation at lower substrate concentration is identical, but in the presence of Triton X-100,

Table 2. PEP-dependent *in vitro* phosphorylation of glucose

K_m values for the substrate glucose and the K_i values for the determined inhibitors of EII^{Glc1} and EII^{Glc2} are shown. Purified EII^{Glc1} and EII^{Glc2} were used in the EII^{Glc} activity test and the substrate-dependent kinetics of glucose phosphorylation were determined in the presence of different inhibitor concentrations (see Methods). The K_m values, which are 12 μ M for EII^{Glc1} and 19 μ M for EII^{Glc2} in the membrane-bound form, were slightly increased by solubilization of the enzymes. –, No inhibition.

Inhibitor	K_i (μ M)	
	EII ^{Glc1} (K_m 30.5 μ M)	EII ^{Glc2} (K_m 37.2 μ M)
2-Deoxyglucose	39.7	–
Methyl α -D-glucoside	–	34.9
Methyl β -D-glucoside	9.4	21.8
Salicin	–	17.9
<i>p</i> -Nitrophenyl α -D-glucoside	–	32.1
<i>o</i> -Nitrophenyl β -D-glucoside	–	28.5

the activity at higher glucose concentrations, and thus the V_{max} value, increases up to 120% (Fig. 2), resulting in an increased K_m value. One possible reason for this may be the existence until solubilization of closed membrane vesicles, which may be rate-limiting in cellular membrane suspensions (Lengsfeld *et al.*, 1973), thus slightly changing the apparent kinetic properties of the EII proteins.

For determination of inhibition constants, the influence of different carbohydrates on the glucose-phosphorylation reaction by purified EII proteins was investigated. In the case of an inhibition, the kinetics of the glucose phosphorylation were measured in the presence of different inhibitor concentrations, and the K_i values were calculated by nonlinear curve-fitting. Table 2 summarizes these experiments. The monosaccharides galactose, fructose and mannose, as well as the tested disaccharides cellobiose, sucrose, maltose, lactose, melibiose and trehalose, and also *N*-acetylglucosamine did not show any inhibition of glucose phosphorylation by either EII^{Glc}, and thus seem not to be substrates for either glucose transporter.

In contrast to their similar rate and affinity of glucose transport, the two purified EII^{Glc} proteins showed a different behaviour against glucoside analogues: while glucose transport of EII^{Glc1} was inhibited by 2-deoxyglucose and methyl β -D-glucoside, EII^{Glc2} was inhibited by methyl α -D-glucoside, methyl β -D-glucoside, *p*-nitrophenyl α -D-glucoside, *o*-nitrophenyl β -D-glucoside and salicin. This could be based on a different arrangement of the active-site amino acids, responsible for keeping the substrate in a catalytically relevant position. EII^{Glc1} shows a stronger selection for substitution of the C-1 position. Different interactions of the aglycones with active-site amino acids could be responsible for the

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Fig. 4. Schematic organization of genes and putative elements of regulation in the *glc* locus of *S. carnosus*. *glcA* and *glcB* encode the PTS permeases EII^{Glc}₁ and EII^{Glc}₂, respectively. Sequence analysis of the incomplete ORF upstream *glcA* shows 55 % homology to the antiterminator *glcT* from *B. subtilis*. P_0 , P_1 and P_2 are putative promoters; CRE 1, CRE 2 and CRE 3 are putative catabolite-responsive elements. SD indicates a putative Shine-Dalgarno sequence and RAT a putative antiterminator binding site. The arrows indicate the relative positions of sites used for DNA manipulations to construct the promoter deletions *glcA*- ΔP_0 (1), *glcA*- ΔP_1 (2 and 3) and *glcA*- ΔP_{01} (3).

mination loop, this indicates independent expression of *glcA* and *glcB*.

To prove the ability to initiate transcription of *glcA*, the two upstream promoters P_0 and P_1 were deleted as described above. After expression of the constructs in *E. coli* WA2127 $\Delta ptsG::Cm^r$, cell membranes showed impaired *in vitro* glucose phosphorylation compared to *glcA* (*glcA*- ΔP_0 , 25 %; *glcA*- ΔP_1 , 63 %; *glcA*- ΔP_{01} , 5 %, Fig. 2). The residual activity of *glcA*- ΔP_{01} can be explained by transcription products initiated at the upstream *bla* promoter of the pUC18 vector combined with its high copy number. A control plasmid lacking both promoters, the ribosome-binding site and the codons of the first 38 amino acids of EII^{Glc}₁ caused no detectable glucose phosphorylation (data not shown).

Thus, both promoters, P_0 and P_1 , are able to initiate the transcription of *glcA* in the heterologous host *E. coli*. P_0 shows a higher activity (63 %) than P_1 (25 %). We expect both putative promoter regions to be involved in regulation of EII^{Glc}₁ expression in *S. carnosus*, in which during growth on glucose the EII^{Glc} activity of membrane fractions increases about five- to sevenfold (data not shown). Although catabolite repression via CRE elements and RAT specific antitermination is not probable in the heterologous host *E. coli*, it cannot be ruled out that these elements influence the residual promoter activities. CRE1 is missing in all three constructs ΔP_0 , ΔP_1 and ΔP_{01} ; the putative RAT sequence is only absent in ΔP_{01} . To confirm the role of P_0 and P_1 , as well as the role of the existence of two EII^{Glc} coding genes in regulation of glucose uptake in *S. carnosus*, further work should focus on genetical analysis in the homologous organism, e.g. disruption of genes or regulative elements, following the expression pattern using reporter genes.

Catabolite repression in Gram-positive bacteria is mediated by HPr after ATP-dependent phosphorylation of HPr on a seryl residue. Recently, the genes of the HPr kinase *hprK* and the phosphatase *hprP* have been identified in *E. coli* (Galinier *et al.*, 1998; Reizer *et al.*, 1998). A complex of CcpA, HPr-Ser ~ P and, at least in some cases, fructose 1,6-bisphosphate binds to the CRE which is found near the transcription start of many catabolic genes, resulting in repression of their expression (Hueck *et al.*, 1994).

Sequence analysis revealed the existence of three CRE-like elements in the region of *glcA* and *glcB* (Fig. 4), suggesting that binding of a CcpA/HPr-Ser ~ P to these regions leads to an additional control of the PTS. One possibility is a negative regulation to protect cells from a toxic effect of accumulated sugar phosphates.

Also of interest is the finding of a partially sequenced ORF preceding *glcA* (Christiansen & Hengstenberg, 1996), encoding the C-terminus of a protein with mean amino acid identity of 25 % and similarity of 55 % to the corresponding parts of bacterial antiterminators of the BglG family (data not shown). So far, these genes have been found in β -glucoside operons of *E. coli* (Schnetz & Rak, 1990) and *Erwinia chrysanthemi* (El Hassouni *et al.*, 1992), as well as in the *sac*, *lev* and *lic* operons of *B. subtilis* (Crutz *et al.*, 1990; Débarbouillé *et al.*, 1990; Schnetz *et al.*, 1996). Their antiterminator proteins bind to the RAT region of mRNA, preceding a terminator stem-loop, resulting in antitermination. By PTS-dependent phosphorylation the antiterminators are regulated in their activity, resulting in adaptation to the environmental conditions.

Recently, a regulation of the *ptsG* gene in *B. subtilis* via antitermination was described following the presented mechanism (Stülke *et al.*, 1997). In *S. carnosus* a stem-loop (TAACTAATTCGATTAGGCATGAGTGA) with 85 % identity to the glucose-specific RAT sequence of *B. subtilis* is located between the putative *glcT* gene and *glcA*, followed by a putative weak termination loop (AAGTTTGAGCAATCCAACCTTTT).

In conclusion, we have cloned a genomic region of *S. carnosus* encoding two very similar PTS permeases with the common substrate glucose but a clearly distinguishable substrate specificity towards glucosides. The identified and putative regulatory elements suggest a complex regulatory mechanism of glucose metabolism involving differential gene expression, transcriptional regulation, catabolite repression and antitermination.

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