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The Different Functions of BglF, the *E. coli* β-Glucoside Permease and Sensor of the *bgl* System, Have Different Structural Requirements†

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ABSTRACT: The *Escherichia coli* BglF protein (EII^{bgl}) is an Enzyme II (EII) of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) which catalyses transport and phosphorylation of β-glucosides. In addition to its transport function, BglF serves as a β-glucoside sensor which reversibly phosphorylates BglG, the transcription regulator of the *bgl* operon. Like many other PTS sugar permeases, the BglF protein is composed of three discrete functional and structural domains: IIA^{bgl} and IIB^{bgl}, which are hydrophilic, and IIC^{bgl}, which is hydrophobic. The domains of BglF are covalently linked to one another in the order BCA. The IIA^{bgl} domain contains the first phosphorylation site, which accepts a phosphoryl group from the general PTS protein HPr and delivers it to the second phosphorylation site, located in the IIB^{bgl} domain. This second site can deliver the phosphoryl group either to a β-glucoside or to BglG. To elucidate the mechanism by which such different substrates can be phosphorylated by the same active site, we decided to try to separate the different phosphorylation activities catalyzed by BglF. To this end we rearranged the BglF domains and constructed $IICBA^{bgl}$ (scrambled-BglF). Scrambled-BglF behaved like wild-type BglF in its ability to be phosphorylated and to phosphorylate BglG in vitro and in vivo. However, it could not catalyze phosphorylation of β-glucosides in vitro nor their phosphotransfer in vivo, and it could not catalyze BglG dephosphorylation in vitro or in vivo. Therefore, the two reactions induced by β-glucosides, sugar phosphorylation and BglG dephosphorylation, seem to require a specific domain organization: IIB^{bgl} should precede IIC^{bgl}. The order of the B and C domains is irrelevant for BglG phosphorylation, which occurs in the absence of β-glucosides. Because the domain order affects the way that the domains are able to interact, our results suggest that catalysis of the sugar-induced functions depends on specific interactions between IIB^{bgl} and IIC^{bgl}. In light of the previous assumption that domain order in EIIs is immaterial for their function, the finding that the order of the domains is important for the function of BglF as a sugar phosphotransferase raises two possibilities: (a) BglF differs from other EIIs in this regard; (b) BglF represents a subgroup of EIIs in which the requirement for a specific domain order correlates with the ability to transport a set of structurally related sugars.

BglF (EII^{bgl})[1] of *Escherichia coli*, is a member of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS), which catalyzes concomitant transport and phosphorylation of β-glucosides (*1*). BglF also controls the activity of a transcription regulator, BglG, by phosphorylating and dephosphorylating it, depending on β-glucoside availability (*2*−*4*). Reversible phosphorylation of BglG regulates its activity as a transcription antiterminator by modulating its dimeric state (*5*). Together, BglF and BglG constitute a novel sensory system which regulates expression of the β-glucoside-utilization operon, *bgl* (*6*). In the absence of β-glucosides, BglF phosphorylates BglG. BglG∼P is an inactive monomer that cannot interrupt premature transcription termination of the *bgl* operon. In the presence of β-glucosides, BglF dephosphorylates BglG∼P, allowing it to dimerize and function as an antiterminator for *bgl* operon transcription.

The phosphate flux in the PTS starts with a phosphoryl group donated by PEP. The phosphate is passed through the general PTS proteins, enzyme I (EI), and HPr, to the different sugar-specific permeases (enzyme II complexes or EIIs). A typical EII consists of three functional domains. IIA is hydrophilic and possesses the first phosphorylation site, a conserved histidine that can be phosphorylated by HPr∼P. IIB is also hydrophilic and possesses the second phosphorylation site, usually a conserved cysteine that accepts the phosphoryl group from IIA∼P and delivers it to the incoming sugar. IIC is hydrophobic and contains transmembrane helices that presumably form the sugar translocation channel and at least part of the sugar-binding site (reviewed in ref *7* and ref *8*). The three EII domains are not necessarily all parts of the same polypeptide. The number of polypeptide chains and the order of the domains

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[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; HPr, histidine-containing phosphocarrier protein; EII^{bgl}, β-glucoside-specific enzyme II; EII^{glc}, glucose-specific enzyme II; EII^{nag}, *N*-acetylglucosamine-specific enzyme II; ATP, adenosine 5′-triphosphate; MBP, maltose binding protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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can differ from one EII to another. Thus, the evolution of different EIIs seems to be the result of domain splitting and splicing. Because all EIIs catalyze sugar transport and phosphorylation, it has been suggested that the order of the domains is largely immaterial to their function (*7*).

BglF contains the three domains described above on the same polypeptide in the order BCA (IIBCA^{bgl}). BglF is the first EII shown to reversibly phosphorylate a transcription regulator in addition to phosphorylating its sugar substrate. The same active site on BglF, Cys 24 in the IIB^{bgl} domain, delivers the phosphoryl group to the two substrates, β-glucosides and BglG (*9*). To analyze the different functions catalyzed by BglF, we shuffled its three domains to construct a BglF derivative which contains the domains in the order CBA. We call this rearranged protein scrambled-BglF. We show here that scrambled-BglF behaves like wild-type BglF in its ability to be phosphorylated, to form dimers, and to phosphorylate BglG. However, it does not catalyze the phosphorylation of β-glucosides nor dephosphorylate BglG in the presence of the sugar. In vivo, scrambled-BglF prevents BglG from acting as an antiterminator, but it does not reverse this effect upon the addition of the sugar. It also cannot complement strains lacking a functional *bglF* gene. We conclude that the two activities of BglF, which are coupled, sugar phosphorylation and BglG dephosphorylation, can be catalyzed only when the IIB domain precedes the IIC domain, although BglF can phosphorylate BglG regardless of the order of its domains.

EXPERIMENTAL PROCEDURES

Strains. The following *E. coli* K12 strains were used: K38 (HfrC *trpR thi* λ^+) was obtained from C. Richardson. LM1 contains mutations in the *nagE* and *crr* genes, which code for II^{nag} and IIA^{glc}, respectively (*10*). AE304-4 and MA200-1 both carry a defective *bglF* gene; the latter also carries a chromosomal *bgl′*−*lacZ* fusion (*11*). PPA547 is a derivative of MA200-1 which contains additional mutations in the *crr* and *nagE* genes (*9*). The *S. typhimurium* strain LJ144 contains the *pstHI-crr* genes on an *E. coli* plasmid, F′198, and thus produces increased levels of EI, HPr, and IIA^{glc} (*12*).

Plasmids. Plasmids pT712 and pT713 contain the phage T7 late promoter, and plasmid pGP1-2 carries the T7 RNA polymerase gene under the control of the lambda CI857 repressor (Bethesda Research Laboratories). Plasmid pT7FH-G carries the entire *bglG* gene cloned downstream of the T7 promoter in pT713; plasmid pT7OAC-F carries the entire *bglF* gene cloned downstream of the T7 promoter in pT712 (*2*). Plasmid pT7CQ-F9 codes for a BglF derivative with longer linkers between the domains (this protein, designated BglF-like, is 633 amino acids long; see Figure 1) cloned downstream of the T7 promoter (*13*). Plasmids pT7CQ-F3 and pT7CQ-F6 encode IIB^{bgl} and IICA^{bgl}, respectively, cloned downstream of the T7 promoter (*13*). Plasmid pT7CQ-F7 encodes IICBA^{bgl} (645 amino acids long, designated scrambled-BglF; see Figure 1) cloned downstream of the T7 promoter. It was constructed as follows: a 249 bp *Bam*HI−*Pst*I fragment encoding the B domain of BglF was prepared from pT7CQ-F3. The *Bam*HI site was subsequently converted to a PstI site by the use of a synthetic linker (5′GATCCTGCAG3′). The modified fragment was inserted into plasmid pT7CQ-F6 at the *Pst*I site, which is located in the region encoding the linker between the C and A domains of BglF.

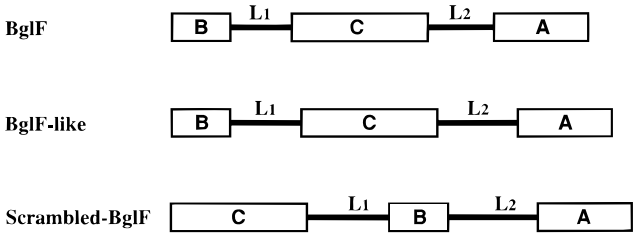

FIGURE 1: Schematic representation of the different BglF derivatives. BglF-like protein and scrambled-BglF were constructed as described in Experimental Procedures. Open squares represent the BglF domains; A = IIA^{bgl}; B = IIB^{bgl}; C = IIC^{bgl}; L1 = Linker 1; L2 = Linker 2.

Chemicals. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Rotem Industries LTD (Israel). [^{35}S]Methionine (1200 Ci/mmol) was obtained from Du Pont. PEP, pyruvic acid, and pyruvate kinase were obtained from Sigma. [^{32}P]PEP was prepared and separated from [^{32}P]ATP as described previously (*2*). MBP−BglG (BglG fused to maltose-binding protein), which can be readily purified on an amylose column, was prepared as described previously (*9*). Purified enzyme I and HPr were obtained from Dr. J. Reizer.

Molecular Cloning and β-Galactosidase Assay. All manipulations with recombinant DNA were carried out by standard procedures (*14*). Assays for β-galactosidase activity were carried out as described by Miller (*15*). Cells were grown in minimal medium which was supplied with 0.4% succinate as carbon source.

[^{35}S]Methionine-Labeling of BglF Derivatives. K38 cells containing plasmid pGP1-2 and one of the plasmids carrying the *bglF* gene or its derivatives under the control of the phage T7 promoter (pT7OAC-F, pT7CQ-F7, pT7CQ-F9) were induced and labeled with [^{35}S]methionine in the presence of rifampicin (Sigma) as described previously (*16*). To study the stability of plasmid-encoded BglF derivatives, unlabeled methionine was added to a final concentration of 0.5 mg/mL to the growth medium (chase) following 2 min of pulse labeling with [^{35}S]methionine, and aliquots were removed at various times for autoradiographic analysis. To study the oligomeric state of the BglF derivatives, the [^{35}S]-labeled proteins were mildly extracted by incubation at 30 °C for 30 min in electrophoresis sample buffer (see below) as described previously (*13*).

Preparation of Cell Extracts and Membrane Fractions. Cell extracts enriched for BglG and membrane fractions enriched for the various BglF derivatives (wild-type, scrambled-BglF, and BglF-like) were prepared as described previously (*2*). The proteins were expressed from their respective genes cloned under T7 promoter control in plasmids pT7FH-G, pT7OAC-F, pT7CQ-F7, and pT7CQ-F9. Expression of T7 RNA polymerase from plasmid pGP1-2, which is compatible with the pT7 plasmids, was induced thermally. The *E. coli* K38 and LM1 strains were used as hosts. Membrane fractions lacking BglF were prepared from strain LM1/pGP1-2/pT712 and were used as negative controls.

In Vitro Phosphorylation and Dephosphorylation of Proteins. In vitro phosphorylation of the BglF derivatives was carried out essentially as described previously (*9*). Mem-

Table 1: Potential Hinge Sequences in BglF and Its Engineered Derivatives

Linker	BglF derivative	Region connecting domains	Sequence[a]	
L1	Wild type	B and C	68 ADVFLAVNSVAGLDEKAQQAPE	89
L1	BglF-like	B and C	73 ADVFLAVNSVAGTPLDEKAQQAPE	96
L1	Scrambled-BglF	C and B	388 AKRQPAQGAPAGSELARKIVAGVGGA	413
L2	Wild type	C and A	456 RQPAQGAPQEKTPEVITPPEQGGICSP	482
L2	BglF-like	C and A	463 RQPAQGAPAQEKTPEVITPPEQGGICSP	490
L2	Scrambled-BglF	B and A	474 SVAGTPGRPAQEKTPEVITPPEQGGICSP	502

[a] Amino acids which most frequently occur in Q-linkers are underlined. Alanines, prolines, and glycines are dotted underneath.

brane fractions (final protein concentration of 0.9 mg/mL) from LM1 cells overexpressing the different BglF derivatives were labeled by incubation at 30 °C in a mixture containing 10 mg/mL Enzyme I, 40 mg/mL HPr, 10 mM [^{32}P]PEP, and PLB buffer (50 mM Na_2HPO_4, pH 7.4, 0.5 mM $MgCl_2$, 1 mM NaF, and 2 mM dithiothreitol). After incubation for 10 min, reactions were either terminated by the addition of electrophoresis sample buffer containing 5% β-mercaptoethanol or incubated further, as described below. To study dephosphorylation by β-glucosides, salicin was added to a final concentration of 0.2%, and incubation was continued at 30 °C for 5 min. To study BglG phosphorylation, purified MBP−BglG in PLB buffer was added to a final concentration of 10 mM, and incubation was continued at 30 °C for 15 min.

Dephosphorylation of BglG was carried out basically as described previously (*2*). First, a membrane fraction (prepared from K38/pGP1-2/pT7OAC-F) was labeled by incubation at 30 °C for 10 min in a mixture containing [^{32}P]PEP, a soluble fraction of *S. typhimurium* LJ144, and PLB buffer. After addition of a cell extract enriched for BglG (prepared from K38/pGP1-2/pT7FH-G), the incubation mixture was further incubated for 15 min. [^{32}P]BglG was then separated from the [^{32}P]-labeled BglF and PEP and incubated with 0.1% salicin and membranes fractions (at a final protein concentration of 0.5 mg/mL) prepared from strain LM1 containing pGP1-2 and either pT712 or one of the following *bglF* plasmids: pT7OAC-F, pT7CQ-F7, or pT7CQ-F9. Incubation was at 30 °C for various times.

Electrophoresis and Autoradiography. Electrophoresis of proteins was carried out on 10% or 5−12.5% gradient SDS-polyacrylamide gels, as described by Laemmli (*17*). For calibration, prestained midrange protein molecular weight markers (Amersham) were loaded in one lane per gel. All gels were dried and exposed to Kodak XAR-5 X-ray film at −70 °C.

RESULTS

Design of the Scrambled-BglF Protein. The BglF protein (IIBCA^{bgl}) contains three discrete functional and structural domains, IIA^{bgl}, IIB^{bgl}, and IIC^{bgl} (reviewed in ref *8*). The domains are connected by flexible linkers (*18*). We constructed a rearranged BglF derivative in which the order of the domains is CBA (designated scrambled-BglF, or IICBA^{bgl}). The hydrophobic C domain is at the N terminus of scrambled-BglF, and it is followed by the two hydrophilic domains, B and A (Figure 1). The CBA order of domains is very common among sugar permeases of the PTS family (*8*). In designing the recombinant protein, special attention was paid to the peptide linkers between the domains. The connection of the structural domains in a multidomain protein by flexible hinge regions allows each domain to assume its conformation independently of the others.

Several types of such hinge regions have been defined (reviewed in refs *18* and *19*). The Q-linkers, which are rich in the hydrophilic residues glutamine, arginine, glutamate, and semipolar residues such as serine and proline, occur at boundaries of distinct domains of regulatory and sensory transducing proteins (*20*). Q-linkers often connect the IIA domains of PTS EIIs with the IIB and IIC domains (*18*, *21*). AP linkers, found in various proteins, including EIIs of the PTS proteins, are regions rich in alanine, glycine, and proline (*22*) or only alanine and proline (*23*, *24*). The exact composition and length are not critical for either type of linkers (*21*, *23*).

In BglF, the sequence which connects the A domain to the C domain was first predicted to be an AP-linker (*23*), but it was later identified as a Q-linker (*21*). In scrambled-BglF, the A domain is connected to the B domain by a Q-linker, most of which is identical to the original linker in wild type BglF (see Table 1). This sequence is also rich in alanine, proline, and glycine. The other linker in BglF, connecting the B and C domains, is rich in residues characteristic of both types of linkers; the sequence connecting the C and B domains in scrambled-BglF is also rich in all of these residues (Table 1). To ensure flexibility, the linkers in the scrambled-BglF were made slightly longer than those in wild-type BglF. Another BglF derivative, in which the order of the domains is the same as in wild-type BglF (BCA) but the linkers are slightly longer (designed BglF-like protein; see Table 1 and Figure 1), served as a control in the experiments reported below. We also made sure that the linker connecting the C and B domains in scrambled-BglF is not by any means inferior to linkers connecting the same domains in naturally occurring EIIs in which the C domain precedes the B domain. This property is illustrated by the comparison to the glucose permease (IICB^{glc}) shown in Table 2.

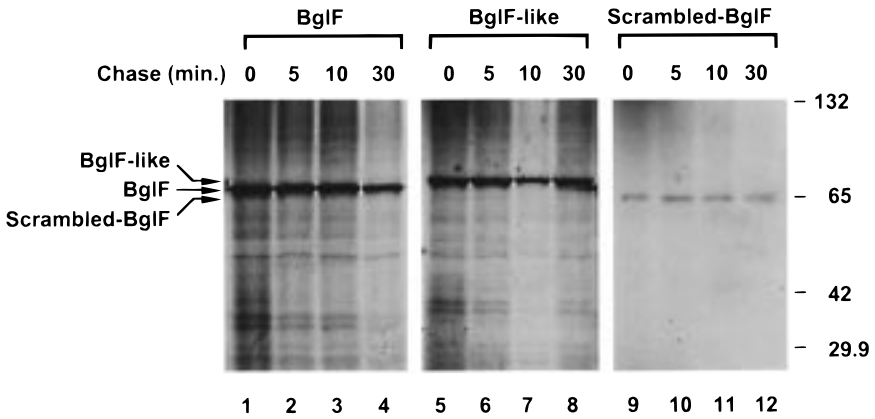

FIGURE 2: The scrambled-BglF protein is stable. Expression of the various BglF derivatives (wild-type, BglF-like, and scrambled-BglF) was induced from plasmids pT7OAC-F, pT7OAC-F9, and pT7OAC-F7, respectively, in *E. coli* K38 cells harboring pGP1-2. The plasmid-encoded proteins were pulse-labeled with [^{35}S]methionine and chased by the addition of unlabeled methionine to the growth medium. Aliquots, removed at the times indicated, were analyzed on 10% SDS−polyacrylamide gels followed by autoradiography. An unstable protein (a truncated BglG protein) was used as a control for the chase success (not shown). Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the position of the various BglF derivatives.

Table 2: Potential Hinge Sequences in the Region Connecting the C and B Domains in Scrambled-BglF and IICB^{glc}

Protein		Sequence[a]	
Scrambled-BglF	386	ITAKRQPAQGAPAGSELARKIVAGVGGA	413
IICB^{glc}	384	PGREDATEDAKATGTSEMAPALVAAFGG	411

[a] Amino acids which most frequently occur in Q-linkers are underlined. Alanines, prolines, and glycines are dotted underneath.

Characteristics of the Scrambled-BglF Protein. The stability of the different BglF derivatives was assayed in pulse-chase experiments. The proteins were labeled with [^{35}S]methionine and chased with unlabeled methionine. Samples were removed at different times and analyzed by SDS−PAGE. The results (Figure 2) demonstrate that the scrambled-BglF protein, although expressed at a lower level than wild-type BglF, is at least as stable as the wild-type protein (Figure 2, compare lanes 9−12 with lanes 1−4). The expression level and stability of the BglF-like protein are very similar to that of wild-type BglF (Figure 2, compare lanes 5−8 with 1−4). The increased mobility of scrambled-BglF may be due to a specific conformation conferred by the CBA domain order, since the migration of hydrophobic proteins on SDS−polyacrylamide gels often differs significantly from what would be predicted solely from the molecular weight.

Next, we tested the ability of the scrambled-BglF and BglF-like proteins to be phosphorylated in vitro. We incubated membranes enriched for the different BglF derivatives with [^{32}P]PEP and purified EI and HPr. The results (Figure 3) demonstrate that the scrambled-BglF and BglF-like proteins can be phosphorylated by the general PTS proteins.

We have recently shown that a fraction of [^{35}S]-labeled and [^{32}P]-labeled BglF can be recovered from the membrane in a dimeric form after mild extraction at 30 °C in the presence of SDS (*13*). The data in Figure 3 show that fractions of the [^{32}P]-labeled scrambled-BglF and BglF-like proteins can also be extracted from the membranes as dimers (Figure 3, D bands in lanes 1−3). We therefore conclude that changing the order of the domains in BglF from BCA to CBA does not affect the ability of the protein to dimerize or to be phosphorylated by the general PTS proteins.

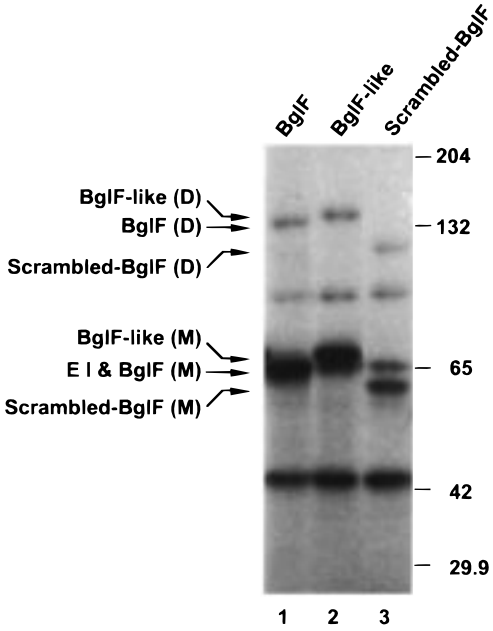

FIGURE 3: The scrambled-BglF can be phosphorylated by the PTS general proteins and can form dimers. Membrane fractions of LM1 cells that overproduce the wild-type BglF, BglF-like, and scrambled-BglF proteins were incubated with [^{32}P]PEP and purified Enzyme I and HPr for 10 min. Proteins were extracted at 30 °C for 30 min in electrophoresis sample buffer. Samples were analyzed on a 5−12.5% gradient SDS−polyacrylamide gel. The autoradiogram is presented. Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the position of monomers (M) and dimers (D) of the various BglF derivatives. Phosphorylated EI (comigrating with wild-type BglF) and IICB^{glc} (monomer, migrating near the 42 Kd molecular weight marker, and dimer) are also detected.

The Order of the Domains in BglF Is Important for β-Glucoside Phosphorylation and BglG Dephosphorylation, but Not for BglG Phosphorylation, in Vitro. We have previously shown that BglF, when phosphorylated in vitro by HPr, can transfer the phosphoryl group either to β-glucosides or to BglG (*2*). The first phosphorylation site of BglF, His 547 in the A domain (site 1), accepts the phosphoryl group from HPr. The phosphoryl group is then

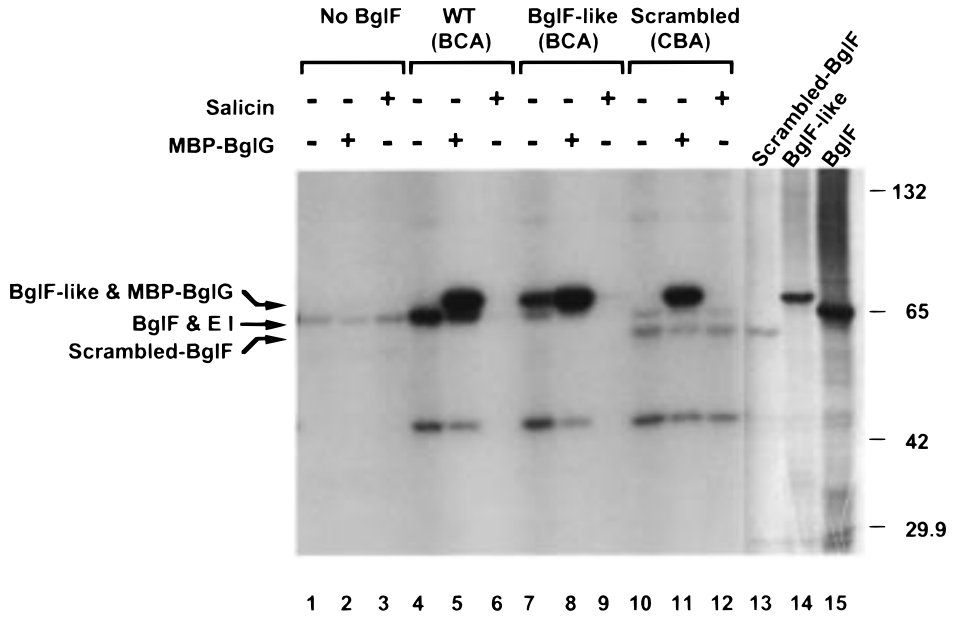

FIGURE 4: The relative order of the IIB and IIC domains in BglF is critical for phosphorylation of β-glucoside but not for phosphorylation of BglG. For the samples in lanes 4−12, the various BglF derivatives were phosphorylated in vitro as described in Figure 3 and incubated with MBP−BglG for 15 min or with 0.2% salicin for 5 min. For the samples in lanes 1−3, membranes of cells not producing BglF were labeled as described in Figure 3 and further incubated with either MBP−BglG or salicin. For the samples in lanes 13−15, the various BglF derivatives were pulse-labeled with [^{35}S]methionine as described in Figure 2. Samples were analyzed on a 5−12.5% gradient SDS−polyacrylamide gel followed by autoradiography. Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the position of the various BglF derivatives, MBP−BglG, and EI. EI and wild-type BglF comigrate in this gel system.

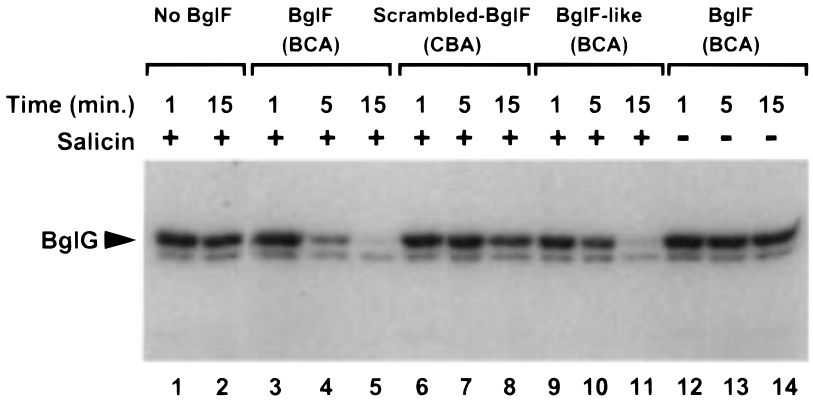

FIGURE 5: The relative order of the IIB and IIC domains in BglF is critical for its ability to dephosphorylate BglG. [^{32}P]BglG, prepared and separated from [^{32}P]BglF, and [^{32}P]PEP as previously described (*2*), was incubated with membrane fractions containing the various BglF derivatives in the presence (+) or absence (−) of 0.2% salicin at 30 °C for the times indicated. Samples were analyzed on a 10% SDS−polyacrylamide gel followed by autoradiography. Molecular masses of protein standards are given in kilodaltons. Arrowhead indicates the position of BglG. The faster-migrating component is a degradation product of BglG (*2*).

transferred to the second phosphorylation site, Cys 24, in the B domain (site 2). The second site delivers the phosphoryl group either to the sugar or to BglG (*9*).

The BglF derivatives, scrambled-BglF, wild-type BglF, and BglF-like protein, were first phosphorylated in vitro as described above (Figure 4, lanes 4, 7, and 10). Purified MBP−BglG (BglG fused to maltose-binding protein), which was previously shown to be phosphorylated by BglF on its BglG moiety in vitro (*9*), was added to the prephosphorylated BglF variants, and the scrambled-BglF was able to phosphorylate it (Figure 4, lane 11). Despite the lower expression level of scrambled-BglF relative to the wild-type BglF and BglF-like proteins (Figure 4, compare lane 13 with lanes 14 and 15), which is reflected in the lower level of phosphorylated scrambled-BglF in the in vitro reactions (Figure 4, compare lane 10 with lanes 4 and 7), it functioned very efficiently as a BglG kinase (Figure 5, compare lane 11 with lanes 5 and 8). This result is not surprising, since BglF had previously been shown to act in a catalytic rather than a stoichiometric way in BglG phosphorylation (*3*). When MBP−BglG was added to membranes of cells that do not produce BglF, which were preincubated with [^{32}P]PEP and purified enzyme I and HPr (Figure 4, lane 1), no phosphorylated MBP−BglG was detected (Figure 4, lane 2). Thus, in agreement with our previously published results (*9*), the phosphorylation of MBP−BglG is not the result of direct phosphotransfer from HPr. These results demonstrate that phosphoryl transfer from site 1 to site 2 of BglF and subsequent delivery to BglG is independent of the order of the IIB^{bgl} and IIC^{bgl} domains.

We next tested the effect of domain order within BglF on its dephosphorylation by β-glucosides. Previous studies demonstrated that dephosphorylation of BglF in the presence of β-glucosides in vitro is a good indicator of the ability to

transfer the phosphoryl group to the sugar (*2*). The β-glucoside salicin was therefore added to the [^{32}P]-prelabeled BglF variants. Unlike the phosphorylated wild-type BglF and BglF-like proteins, which were completely and efficiently dephosphorylated by salicin (Figure 4, lanes 6 and 9, respectively), salicin did not chase the [^{32}P]-label from scrambled-BglF (Figure 4, lane 12). Dephosphorylation of scrambled-BglF was not observed even after extended incubation with the sugar (not shown). We conclude that the second phosphorylation site of scrambled-BglF does not deliver its phosphoryl group to β-glucosides, and if this reaction does occur, it is inefficient. Therefore, a particular order of the IIB^{bgl} and IIC^{bgl} domains (BC), which probably allows for particular interactions between these domains, is needed for BglF to phosphorylate its sugar substrate.

Previous studies demonstrated that β-glucosides stimulate BglF to dephosphorylate BglG∼P (*2*). To test the effect of domain order within BglF on this activity, we incubated [^{32}P]-labeled BglG (purified as described before, ref *2*) with β-glucosides and membranes containing either scrambled-BglF, wild-type BglF, or BglF-like protein. The level of [^{32}P]-BglG decreased in a time-dependent manner, so that after 15 min it could hardly be detected, when it was incubated with the β-glucoside salicin and membranes containing BglF or BglF-like protein (Figure 5, lanes 3−5 and 9−11, respectively). In contrast, no significant dephosphorylation of [^{32}P]BglG was observed when it was incubated with membranes containing scrambled-BglF in the presence of salicin (Figure 5, lanes 6−8). The result with scrambled-BglF resembled those obtained when [^{32}P]BglG was incubated with BglF alone (Figure 5, lanes 12−14) or with salicin alone (Figure 5, lanes 1−2). The possibility that the inability of scrambled-BglF to dephosphorylate BglG∼P is due to its low concentration relative to the wild-type BglF and BglF-like proteins was ruled out, since increasing the amount of membranes containing scrambled-BglF in the incubation mixture 5-fold did not reduce the level of [^{32}P]BglG (data not shown). Therefore, only BglF derivatives in which the IIB domain precedes the IIC domain (from N-terminus to C-terminus) seem to be able to fold into the conformation which can catalyze efficient dephosphorylation of BglG∼P in the presence of β-glucosides.

The BC Domain Order Is Required for BglF To Mediate β-Glucoside Utilization and Restore Sugar-Induced BglG Antitermination, but Not for Its Activity as BglG Negative Regulator, in Vivo. To substantiate our in vitro results, we tested in vivo whether a plasmid which encodes scrambled-BglF can complement bglF mutant strains. Complementation was indicated by the formation of red colonies on MacConkey arbutin plates. Utilization of the β-glucoside arbutin depends on the ability of the plasmid-encoded BglF derivatives to phosphorylate and transport this sugar, which is then cleaved by the product of the unlinked locus *bglA*. Utilization of the β-glucoside salicin is prohibited in these strains due to the polarity of the mutation in *bglF* on the cotranscribed *bglB* gene, whose product preferentially cleaves phosphosalicin (*11*). The results are presented in Table 3. Plasmid-encoded scrambled-BglF could not complement the *bglF* strains, whereas the plasmid-encoded wild-type BglF and BglF-like proteins complemented the *bglF* strains. Indeed, the level of scrambled-BglF in the cell might be lower than the levels of the other two BglF variants, as suggested by the results which are presented in Figure 2. However, in this experiment, the BglF variants, including scrambled-BglF, were expressed from multicopy plasmids, whereas the level of BglF encoded from a single copy of a chromosomal gene is sufficient for efficient β-glucoside utilization. Therefore, these observations indicate that scrambled-BglF does not catalyze transport of β-glucosides, as predicted from the in vitro data, and if it does, it is not efficient enough to enable complementation of *bglF* mutant strains.

Table 3: Biological Functions of Scrambled-BglF in Vivo

plasmid	plasmid-encoded BglF derivative	domain order	complementation[a] of *bglF* mutant strains[b]	β-gal activity (U)[c]	
				−salicin	+salicin[d]
pT7OAC-F	wild type	BCA	+	5 ± 1	36 ± 10
pT7CQ-F9	BglF-like	BCA	+	6 ± 1	21 ± 6
pT7CQ-F7	scrambled-BglF	CBA	−	5 ± 0.5	6 ± 1
pT712	−		−	83 ± 24	95 ± 18

[a] Complementation was indicated by colony color on MacConkey arbutin plates: (+) strong complementation (bright red colonies); (−) no complementation (white colonies). [b] Complementation of strains AE304-4 and MA200-1 was assayed. [c] β-Galatosidase activity was assayed in strain MA200-1, which carries a *bgl′−lacZ* transcriptional fusion and a defective *bglF* gene. The values represent the average of four independent measurements. [d] Salicin (7 mM) was added to the growth medium.

The ability of BglF to inactivate the transcriptional antiterminator BglG in the absence of β-glucosides stems from its ability to phosphorylate BglG. The relief of BglG inhibition upon β-glucoside addition is due to dephosphorylation of BglG∼P by the sugar-stimulated BglF (*2*). To examine the ability of scrambled-BglF to phosphorylate and dephosphorylate BglG in vivo, we made use of strain MA200-1, which carries a chromosomal fusion of the *bgl* promoter and transcriptional terminator to *lacZ* and lacks a functional *bglF* gene. BglG is not negatively regulated by phosphorylation in this strain, and high β-galactosidase activity is measured whether or not β-glucosides are added to the growth medium (Table 3, last line). Expression of plasmid-encoded wild-type BglF in strain MA200-1 renders *lacZ* expression inducible; in the absence of β-glucosides, the *lacZ* gene in not transcribed because BglG is inactivated by phosphorylation, and upon addition of β-glucosides BglF dephosphorylates BglG, allowing it to block transcription termination and the *lacZ* gene in expressed (Table 3, first line). Plasmid-encoded BglF-like protein behaved like wild-type BglF in regulating BglG activity in strain MA200-1 (Table 3, second line). In strain MA200-1 producing plasmid-encoded scrambled-BglF, the β-galactosidase activity was low when grown in the absence or presence of β-glucosides (Table 3, third line), indicating that scrambled-BglF can inhibit BglG activity but cannot lift the inhibition. These results are consistent with our in vitro results which show that scrambled-BglF can phosphorylate BglG but not dephosphorylate it upon addition of β-glucosides.

DISCUSSION

The complex action of BglF as the β-glucoside phosphotransferase and sensor of the *bgl* system provides an

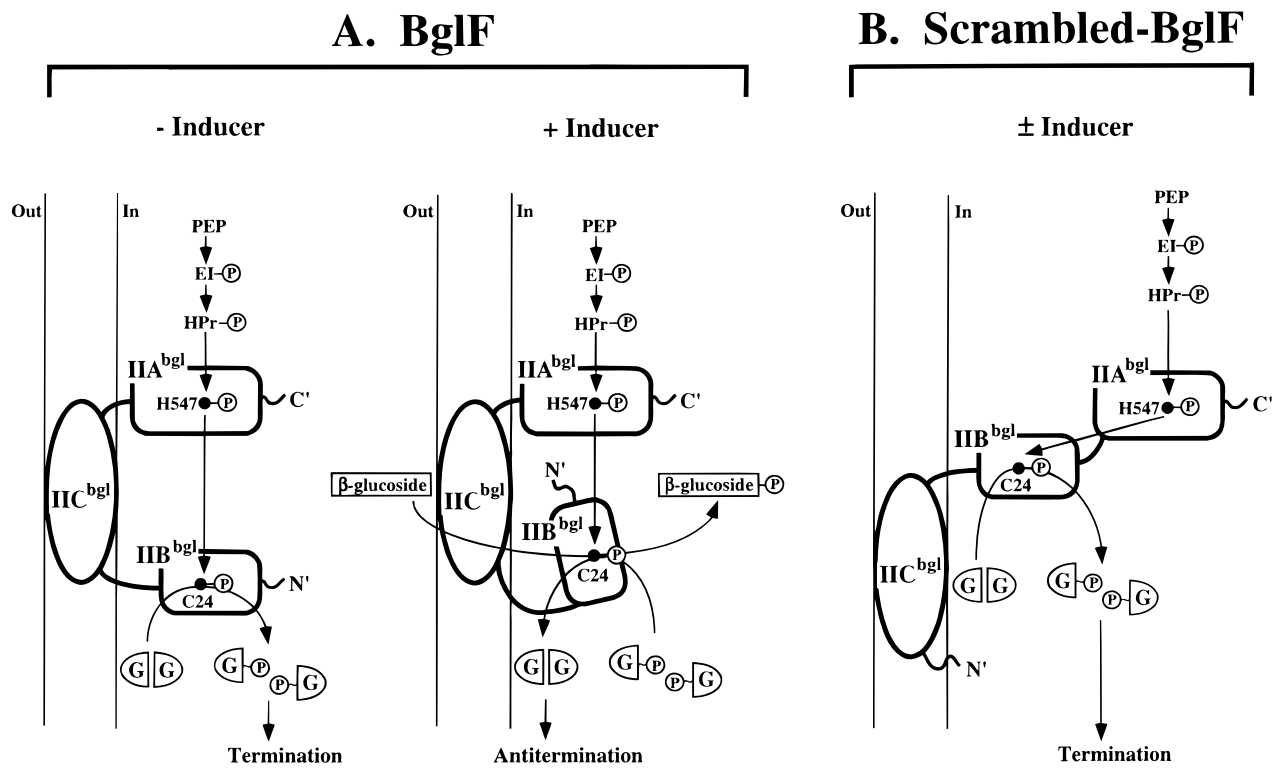

FIGURE 6: Model for the implications of domain order on BglF function. A sugar-induced interaction between the IIB^{bgl} and IIC^{bgl} domains, which depends on their relative positions, might occur in wild-type BglF but be precluded in scrambled-BglF.

opportunity to study the structural basis for the catalysis of stimuli-induced versus uninduced functions. The phosphorylation reactions catalyzed by BglF are interdependent and mutually exclusive: in the absence of β-glucosides, BglF phosphorylates BglG; upon β-glucoside addition, BglF dephosphorylates BglG and phosphorylates the sugar. The same active site on BglF delivers the phosphoryl group to the sugar and to BglG (*9*). It is logical to assume that different sequences and/or different conformations of BglF are required for the different phosphorylation functions.

We have recently demonstrated that BglF dimerizes, but its dimeric state is not modulated by β-glucosides (*13*). Rather, BglF dimerization in the membrane seems to occur spontaneously, and the dimers can catalyze both sugar and BglG phosphorylation. Therefore, modulation of BglF dimerization is not the key to the sugar-controlled differential activity of BglF.

To unravel the basis for differential phosphorylation by BglF, it is important to elucidate the requirements of the different functions. The structural requirements for β-glucoside phosphorylation and BglG dephosphorylation, both of which are stimulated by the sugar, are apt to be similar to each other and to differ from the requirements for BglG phosphorylation, which occurs in the absence of sugar. In the experiments reported here, we uncoupled the reactions catalyzed in the presence of sugar from those catalyzed in the absence of sugar. We found that the order of the domains in BglF (BCA) is important for β-glucoside phosphorylation and BglG dephosphorylation, whereas BglG phosphorylation can be catalyzed by a scrambled-BglF derivative with the domain order BCA. The requirement for a specific domain order to catalyze the sugar-stimulated functions has several possible causes. An area in BC^{bgl} that includes the junction between these domains might be required, or a conformation conferred by this order might be recognized by the sugar. A likely mechanism, shown in Figure 6, is that an interaction between the IIB^{bgl} and IIC^{bgl} domains, which depends on their relative positions, might be induced by β-glucosides. Such an interaction could induce a conformational change in BglF and might be the key to the sugar-induced signal transduction pathway that leads to the expression of the *bgl* operon. A major difference between scrambled-BglF and wild-type BglF is that in the first, the B domain is adjacent to the C-terminal part of the C domain, whereas in the second it is a adjacent to the N-terminal part of the C domain. Hence, the sugar-induced interaction between the B and C domains that can occur in wild-type BglF might be precluded in scrambled-BglF.

The mechanism by which β-glucosides stimulate BglF to dephosphorylate BglG$\sim$P is not understood. One possibility is that phosphorylation of BglG by BglF is a reversible reaction, and the sugar substrate, by dephosphotylating BglF$\sim$P, shifts the equilibrium, thus leading to dephosphorylation of BglG$\sim$P. If this is the case, the inability of scrambled-BglF to dephosphorylate BglG$\sim$P might stem from its inability to phosphorylate β-glucosides.

Sequence comparisons have shown that most of the PTS permeases derive from a common ancestral protein (reviewed in ref *7*). They constitute a classical example of modular evolution: during the evolution of EIIs the various hydrophobic and hydrophilic domains have either become fused to each other in different orders and combinations or have separated from each other to become distinct polypeptide chains. Because all the EIIs presumably catalyze sugar phosphotransfer by the same mechanism, it has been assumed that the order of the domains within the individual PTS permeases is largely immaterial to their function (*7*). The evidence reported thus far supported this assumption. Linked

domains which were separated by subcloning and then coexpressed retained catalytic activity. When the IIA domain was removed from the linked CB domains of the *E. coli* mannitol permease or the *B. subtilis* glucose permease, IIA could be added back to restore 25% and 100% activity, respectively (*25*−*27*). A free IIA^{bgl} domain also restores the ability of $IIBC^{bgl}$ to phosphorylate both β-glucosides and BglG (*28*). Separation of the B domain of the manitol permease or of the glucose permease from their respective C domains had a more drastic effect, yet catalytic activity was retained to some extent upon their coexpression (*29*−*31*). Moreover, a circularly permuted derivative of the *E. coli* glucose permease, in which the order of the domains is BC rather than CB, as in the wild-type protein, has activity comparable to that of the wild-type protein (*32*). However, separated IIB^{bgl} and IIC^{bgl} domains do not catalyze β-glucoside phosphorylation regardless of whether IIC^{bgl} is covalently linked to IIA^{bgl} or not (*28*). From the results presented in this paper, it is evident that the order of the IIB^{bgl} and the IIC^{bgl} domains is critical for the activity of BglF as a sugar permease. Therefore, the order of the domains in EIIs might not be arbitrary, and in this case it is certainly not.

Sequence analysis of the known EIIs revealed that many of them fall into clusters of related proteins (reviewed in ref *18*). Clustering patterns consistently correlate with sugar specificity, even though the proteins in a cluster may be derived from evolutionarily divergent organisms. Thus, all of the permeases specific for aromatic β-glucosides, sucrose (an α-glucoside), and trehalose (another α-glucoside) form a cluster (*18*). In these permeases, regardless of whether they contain an A domain, the B domain precedes the C domain. This arrangement may simply reflect their evolutionary relatedness. Alternatively, it might be conserved because the recognition of these sugars or the creation of translocation channels for these sugars require it. Does the resistance to domain rearrangement of BglF stem from its complex functions as a kinase and phosphatase of a regulatory protein in addition to the sugar permease activity, or is it a characteristic required for function of all permeases in its evolutionary cluster? Rearrangement of the domain order in another permease of the BglF cluster, which is not involved in regulation, is currently underway. The properties of this scrambled protein should help answer this question.

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