The Localization of the Phosphorylation Site of BglG, the Response Regulator of the Escherichia coli bgl Sensory System*

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BglG, the response regulator of the bgl sensory system, was recently shown to be phosphorylated on a histidine residue. We report here the localization of the phosphorylation site to histidine 208. Localization of the phosphorylated histidine was carried out in two steps. We first engineered BglG derivatives with a specific protease (factor Xa) cleavage site that allowed asymmetric splitting of each prephosphorylated protein to well-defined peptides, of which only one was labeled by radioactive phosphate. This allowed the localization of the phosphorylation site to the last 111 residues. Subsequently, we identified the phosphorylated histidine by mutating each of the three histidines located in this region to an arginine and following the ability of the resulting mutants to be in vivo regulated and in vitro phosphorylated by BglF, the bgl system sensor. Histidine 208 was the only histidine which failed both tests. The use of simple techniques to map the phosphorylation site should make this protocol applicable for the localization of phosphorylation sites in other proteins. The procedure presented here represents a novel family of sensory systems. Thus, the mapping reported here is an important step toward the definition of the functional domains involved in the transduction of a signal by the components that constitute systems of this novel family.

The bgl operon in Escherichia coli, induced by environmental signal (β-glucosides), is regulated by a novel sensory system which consists of a membrane-bound sensor, BglF, and a response regulator, BglG (1). The response regulator is an RNA-binding protein which controls operon expression by transcriptional antiautoregulation (2). The sensor is a phosphotransferase system transport protein which controls the activity of the response regulator by reversible phosphorylation according to β-glucoside availability (3–5). Reversible phosphorylation of the response regulator by reversible phosphorylating according to β-glucoside availability (6). The bgl system is not a member of the known family of two-component regulatory systems involved in signal transduction (reviewed in Refs. 7–10). The bgl proteins, BglF and BglG, share no homology with the sensors and regulators of the two-component systems, respectively. Moreover, it was recently shown that BglG, the response regulator of the bgl system, is phosphorylated on a histidine residue, unlike response regulators of the two-component systems which are phosphorylated on an aspartate. Hence, the bgl system represents a novel family of sensory systems. Other systems in different organisms were suggested to affiliate to this new family (11–19). To understand the rules of recognition and interaction between the components which constitute systems of the new family, it is important to define the functional domains involved in the transduction of a signal by these components. Localization and characterization of the dimerization and the phosphorylation sites on BglG is also important for the elucidation of the mechanism by which phosphorylation affects the dimeric state of a protein.

In this paper we report that identification of the phosphorylated residue on BglG, the response regulator of the bgl system, as histidine 208. Mapping of the phosphorylated histidine was carried out in two steps: the first step defined the protein region to which the phosphorylation site maps; the second step defined the specific histidine which is phosphorylated. In the first step we introduced factor Xa cleavage sites to BglG. Proteolysis after in vitro phosphorylation with radioactive phosphate allowed the localization of the phosphorylation site to the last 111 residues of the protein. In the second step we mutated the three histidines located in this region, each at a time, to arginines and followed the ability of the mutants to be in vivo regulated and in vitro phosphorylated by BglF. The ability of all the BglG mutant derivatives used in this study to fold correctly was deduced from various assays that monitored their ability to function as transcriptional antiterminators. Our protocol for the localization of the BglG phosphorylation site can be adopted and applied to localize phosphorylation sites of other proteins.

EXPERIMENTAL PROCEDURES

Strains—The following E. coli K12 strains were used: K38 (HfrC thi λ−); AE304-7 and AE304-9, both carry a defective bglG gene and an activating mutation in bglF (20); MA152 and MA200, both carry a bgl–lacZ fusion on their chromosome (λ bglR7 bglG lacZ‘ lacY‘), but while the first is Δbgl, the second is bgl+ (20). Salmonella typhimurium strain L1144 (cpp-401, cysA1150/F9), carries the pts operon on an E. coli plasmid, F’198, and thus produces increased levels of Enzyme I, HPr, and Enzyme IIA (21).

Plasmids—Plasmids pT712 and pT713, containing the phase T7 late promoter, and plasmid pGP1-2, carrying the T7 RNA polymerase gene under control of the ACIB5 repressor, were obtained from Life Technologies, Inc. Plasmid pT7OAC-F carries the entire bglF gene cloned downstream of the T7 promoter in pT712; plasmid pT7FH-G carries the entire bglG gene cloned downstream of the T7 promoter in pT713 (3). Plasmid pMN25 carries the entire bglG gene cloned in plBR322 (20).

The following plasmids were constructed by introducing insertion or base substitution mutations (see the details of the mutagenesis below) into the bglG gene in plasmid pT7FH-G, pCQ-G1 and pCQ-G2 encode for BglG with an insertion of factor Xa cleavage site, Ile-Glu-Gly-Arg,

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between amino acids 113 and 114 and amino acids 167 and 168, respectively. pCQ-G4, pCQ-G5 and pCQ-G6 encode for BglG with His-208, His-219, and His-278 mutated to Arg, respectively.

Media—Enriched media, M9 salts, and M63 salts minimal media were prepared essentially as described by Miller (22). The minimal medium used for \( [\text{H}] \)tryptophan labeling was the same as the one used for \( [\text{S}] \)methionine labeling, except that it lacked tryptophan rather than lacking methionine and cysteine. Ampicillin (200 μg/ml) and kanamycin (30 μg/ml) were included in the media when growing strains containing plasmids that confer resistance to either one of these antibiotics. MacConkey arbutin plates were prepared as described previously (24). MacConkey lactose plates were prepared from lactose MacConkey agar (Difco).

Chemicals—Factor Xa was obtained from New England Biolabs. (γ-\( [\text{P}] \))ATP (3000 Ci/mmol) was obtained from Rotem Industries Ltd. (Beer-Sheva, Israel). \( [\text{S}] \)Methionine (1200 Ci/mmol) was obtained from DuPont. \( [\text{H}] \)Tryptophan (33 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. PEP, pyruvic acid, and pyruvate kinase were obtained from Sigma. \( [\text{P}] \)PEP was prepared and separated from \( [\text{P}] \)ATP as described before (3).

Molecular Cloning—All manipulations with recombinant DNA were carried out by standard procedures (25). Restriction enzymes and other enzymes used in recombinant DNA experiments were purchased commercially and were used according to the specifications of the manufacturers.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out by overlap extension with polymerase chain reaction as described by Ho et al. (26). To mutate the bglG gene in plasmid pT7FH-G to its alleles that encode for BglG derivatives containing the factor Xa cleavage site insertion (Ile-Glu-Gly-Arg), the following primers were used: pEP, pyruvic acid, and pyruvate kinase were obtained from Sigma. \( [\text{P}] \)PEP was prepared and separated from \( [\text{P}] \)ATP as described before (3).

Localization of BglG-phosphorylated Histidine—Localization of the Region on BglG which Contains the Phosphorylation Site—Previous studies have demonstrated the existence of two forms of BglG in vivo, phosphorylated and non-phosphorylated, implying that there is a single phosphorylated species of BglG (4). The phosphorylated residue on BglG was identified as a histidine. As a first step toward the localization of the phosphorylated histidine on BglG, we attempted to identify the region on BglG that contains this histidine. To do that, we took advantage of the idea that a cleavage site for a specific protease can be inserted into proteins by in vitro manipulations (31). Our plan was to engineer BglG mutants, each containing a factor Xa site located asymmetrically in the protein, to enable cleavage of the mutant proteins to two fragments of different sizes. Cleavage of these mutant BglG derivatives following their in vitro phosphorylation is expected to give only one \( [\text{P}] \)P-labeled fragment in each case, due to the phosphorylation of BglG on a single residue. The ability of the engineered BglG derivatives to fold correctly, dimerize, bind RNA, and antiter-
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FIG. 1. Experimental design for the definition of the region on BglG that contains the phosphorylation site. G, BglG; MG1 and MG2, BglG mutants with an internal insertion of factor Xa cleavage site; filled boxes represent the factor Xa cleavage sites; the three asterisks represent the three histidines located in the carboxyl-terminal 111 amino acids.

FIG. 2. BglG phosphorylation site resides in its last 111 residues. The different BglG derivatives (wild type, MG1, and MG2), labeled in vivo with [³⁵S]methionine or phosphorylated in vitro by [³²P]BglF, were purified from an SDS-polyacrylamide gel and treated with factor Xa as described under “Experimental Procedures.” The resulting polypeptides and the untreated purified proteins were separated on a Tricine-SDS-polyacrylamide gel. An autoradiogram of the gel is shown. The untreated [³⁵S]MG2 and [³²P]MG2 in lanes 6 and 7 are shown as representatives for the purity of all gel-purified BglG derivatives. Arrowheads indicate the positions of BglG and its mutants MG1 and MG2, as well as the proteolysis products of MG1 (a and b fragments) and MG2 (c and d fragments). Molecular masses of protein standards are given in kilodaltons.

In addition, incubation of both wild-type BglG and MG1 with factor Xa resulted in nonspecific cleavage that generated slightly shortened derivatives of these proteins (Fig. 2, lanes 1 and 2). However, this did not interfere with the identification of the specific cleavage products, a and b. In vitro phosphorylation of MG1 prior to proteolysis with factor Xa resulted in the appearance of one [³²P]-labeled fragment, the b fragment (Fig. 2, lane 3). To indisputably identify the slower migrating fragment as polypeptide b, the C-terminal fragment, we took advantage of the fact that all tryptophans in BglG are clustered in fragment b. Thus, cleavage of BglG, which was labeled with [³¹H]tryptophan, by factor Xa is expected to generate only one labeled fragment, the b fragment. The results presented in Fig. 3 demonstrate that indeed [³¹H]tryptophan-labeled MG1 generates only one labeled fragment, the slower migrating fragment, after incubation with factor Xa (Fig. 3, lane 2). This is in contrast to the two labeled polypeptides produced by factor Xa proteolysis of [³⁵S]MG1 (Fig. 3, lane 1). Thus, this experiment verifies the identification of the slower migrating fragment as polypeptide b. The results obtained with MG1 indicate that the phosphorylation site resides in the last 165 amino acids of BglG. The possibility that MG1 is misfolded and thus phosphorylated differently from wild type was ruled out by the demonstrated ability of MG1 to complement bglG strains the same as wild type (Table I).

We next constructed MG2 that contains a factor Xa site in a position different from MG1. This was accomplished by introducing a factor Xa site between amino acids 167 and 168. Cleavage of MG2 with factor Xa is expected to generate two fragments, “c” and “d,” consisting of 171 amino acids and 111 amino acids, respectively (see Fig. 1). Incubation of [³⁵S]-labeled MG2 with factor Xa resulted indeed in the appearance of two labeled fragments with molecular masses corresponding to those expected for peptides c and d (Fig. 2, lane 4). Proteolysis of in vitro phosphorylated MG2 with factor Xa yielded one [³²P]-labeled fragment, the d fragment (Fig. 2, lane 5). MG2, like MG1, strongly complemented bglG strains (Table I). Based on the results with MG1 and MG2, we could conclude that the phosphorylation site resides in the last 111 amino acids of BglG.

Mapping the Phosphorylated Amino Acid on BglG—We subsequently constructed three mutants of BglG, each with one of the three C-terminal histidines of BglG (His-208, His-219, and His-278, marked as asterisks in Fig. 1) mutated to an arginine, to generate MG4 (H208R), MG5 (H219R), and MG6 (H278R). These mutants were checked for their ability to complement bglG strains (Table I). The plasmid-encoded MG4, MG5, and MG6 mutants strongly complemented the chromosomal mutation in the bglG gene (the same as wild-type BglG) and enabled growth of the mutant strains on β-glucosides. The results of this analysis suggest that these mutants can form dimers, bind to BglG target site on the RNA, and lead to antitermination of bgl transcription.

To directly demonstrate the ability of the three His to Arg mutants to antiterminate transcription, we made use of strain
MA152 which is deleted for the *bgl* operon and carries a chromosomal *bgl*-lacz fusion (20). The *lacZ* gene is not expressed in this strain, because transcription terminates at the *bgl* terminator, located upstream of the *lacZ* gene. Expression of plasmid-encoded BglG renders the *lacZ* expression in this strain constitutive, due to this protein’s ability to prevent transcription termination. The ability of plasmid-encoded MG4, MG5, and MG6 to antiterminate transcription and enable *lacZ* expression in MA152 was tested by observing the color of the colonies containing these plasmids on MacConkey lactose plates and by measuring the β-galactosidase levels produced by the cells expressing them. As shown in Table II, all three mutants behaved like wild type in their ability to enable *lacZ* expression. These results indicate that all three mutants perform like wild type and antiterminate transcription at the *bgl*-lacz fusion.

After verifying that the three His to Arg mutant proteins do not differ from wild-type BglG in their activity, we aimed at studying their ability to be phosphorylated by BglF. Phosphorylation of BglG by BglF was shown before to be the reason for the negative effect that BglF exerts on BglG activity (3). Thus, as one approach to determine which histidine on BglG is phosphorylated by BglF, we tested the effect of the three missense mutations in BglG on its ability to be negatively regulated by BglF in vivo. To address this question we made use of strain MA200 which carries the same chromosomal *bgl*-lacz fusion as MA152, but is also Bgl⁺ (20). Expression of *lacZ* in this strain is inducible, *i.e.* β-galactosidase is produced only upon the relief of BglF inhibition by the addition of β-glucosides to the growth medium. Expression of a plasmid-encoded BglG mutant that cannot be phosphorylated by BglF renders *lacZ* expression in this strain constitutive, *i.e.* independent of β-glucoside addition. We tested the effect of plasmid-encoded MG4, MG5, and MG6 on *lacZ* expression in MA200 by the same methods employed to study their effect in MA152, *i.e.* colonies color on MacConkey lactose plates and β-galactosidase activity measurements. The results of these tests are summarized in Table III. MG6 behaved like wild-type BglG and did not enable *lacZ* expression in the absence of β-glucosides, while MG4 and MG5 led to constitutive expression of *lacZ*, independent of β-glucoside addition to the medium. Hence, MG4 and MG5 are not subjected to negative regulation by BglF in vivo. This observation suggested to us that the phosphorylated residue on BglG is either His-208 (mutated in MG4) or His-219 (mutated in MG5).

Another approach we applied to determine which of the histidines in BglG is phosphorylated by BglF, was to test the ability of the His to Arg mutants to be phosphorylated by BglF in vitro. We have shown before that BglF, phosphorylated in *vitro* in the presence of [32P]ATP, Enzyme I, and HPr, can transfer a phosphoryl group to BglG (3). We added extracts of cells producing the different BglG derivatives, wild type and mutants, to mixtures containing prephosphorylated BglF, and further incubated them. Aliquots removed after 1, 5, and 15 min were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography, and the results are presented in Fig. 4. MG6, mutated in histidine 278, was phosphorylated in *vitro* at the same pattern as wild-type BglG (compare lanes 3–5 with lanes 12–14 in Fig. 4). No phosphorylation occurred in the case of MG4, which is mutated in histidine 208 (Fig. 4, lanes 6–8). MG5, mutated in histidine 219, was phosphorylated by BglF, albeit, at a slightly reduced rate than the wild-type protein (Fig. 4, lanes 9–11). A control with BglG incubated in a phosphorylation system lacking BglF (lanes 1 and 2) rules out the possibility of a direct phosphorylation of BglG by the phosphotransferase system general proteins. Hence, MG4 is the only His to Arg mutant that fails to be both in *vivo* regulated and in *vitro* phosphorylated by BglF. These results indicate that histidine 208 is the amino acid which is phosphorylated on BglG.

**DISCUSSION**

The phosphorylated amino acid on the response regulator of the *bgl* system, BglG, was recently identified as a histidine residue.1 In this study we used a combination of biochemical and genetic approaches to map the phosphorylated histidine on

**Table I. Localization of BglG-phosphorylated Histidine**

| Plasmid | Plasmid-encoded BglG derivative | Complementation of *bgl-lacZ* fusion |
|---------|---------------------------------|-------------------------------------|
| pT713   | BglG (wild type)                | +                                   |
| pT7FH-G | MG1 (insertion mutant)          | -                                   |
| pCQ-G2  | MG2 (insertion mutant)          | +                                   |
| pCQ-G4  | MG4 (H208R)                    | +                                   |
| pCQ-G5  | MG5 (H219R)                    | +                                   |
| pCQ-G6  | MG6 (H278R)                    | +                                   |

a The results presented in this table are all with plasmids derived from pT713. Similar results were obtained with pBR322 plasmids containing the different derivatives of the *bgl-lacZ* genes (wild type and the five mutants).

**Table II. Ability of BglG mutated in either one of its last histidines to antiterminate transcription of a chromosomal *bgl-lacZ* fusion**

| Plasmid | Plasmid-encoded BglG derivative | Phenotype on MacConkey-lactose medium | β-Galactosidase activity
|---------|---------------------------------|--------------------------------------|------------------------|
| pT713   | BglG (wild type)                | +                                    | 2                      |
| pMN25   | MG4 (H208R)                    | +                                    | 87                     |
| pCQ-G4  | MG5 (H219R)                    | +                                    | 155                    |
| pCQ-G5  | MG6 (H278R)                    | +                                    | 247                    |

a Expression of the *bgl-lacZ* fusion was partly determined by colony color on MacConkey-lactose plates: +, red colonies; −, white colonies.

b The values represent the average of four independent measurements.

**Table III. The effect of BglF on the antitermination activity of BglG mutants**

| Plasmid | Plasmid-encoded BglG derivative | Phenotype on MacConkey-lactose medium | β-Galactosidase activity
|---------|---------------------------------|--------------------------------------|------------------------|
| pT713   | BglG (wild type)                | +                                    | 2                      |
| pMN25   | MG4 (H208R)                    | +                                    | 87                     |
| pCQ-G4  | MG5 (H219R)                    | +                                    | 155                    |
| pCQ-G5  | MG6 (H278R)                    | +                                    | 247                    |

a Phenotypes on the MacConkey-lactose plates are represented as in Table II.

b The values represent the average of four independent measurements.

c β-Methylglucoside (10 mM) was used as the inducer.

d pBR322 behaved as pT713 (not shown).
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BglG. We first defined the protein region to which the phosphorylation site maps by introducing a protease cleavage site to two locations in the protein, enabling its proteolysis to well defined fragments. Phosphorylation of a protein containing an engineered cleavage site with radioactive phosphate followed by its proteolysis results in the appearance of one radioactively labeled fragment, the one on which the phosphorylated residue resides. Hence, by constructing two mutant BglG proteins (MG1 and MG2), each containing a factor Xa cleavage site at a different location, we could locate the phosphorylated histidine to last 111 residues. This part of the protein contains three histidines, which we mutated, each at a time, to arginine, the residue which is most similar to histidine. To determine which histidine is the phosphorylated one, we followed the mutants’ ability to be negatively regulated by BglF and therefore concluded that histidine 208 constitutes the phosphorylation site, enabled phosphorylation of the mutant protein by BglF. We hypothesized that histidine 208 as BglG phosphorylation site suggests that these mutations are in other sites involved in BglG-negative regulation and phosphorylation. Whether they are part of the site which is recognized by the kinase and interacts with it or they are part of a phosphorylation module composed of residues remote from each other in the primary sequence but brought to close vicinity during folding awaits future structural studies.

Ample precautions were taken to ensure that the histidine to arginine mutants are otherwise identical to the wild-type protein. These proteins were shown to strongly complement a mutation in the bglG gene and enable β-glucoside utilization. Their transcriptional antitermination activity was also demonstrated directly by their ability to express a lacZ gene fused downstream to the bglI transcriptional terminator. As part of the precautions, this ability was assayed in the different bacteria. Passing all these hurdles requires that the mutant proteins fold, dimerize, bind RNA, and antiterminate transcription the same as the wild-type protein. These strict demands for the performance of the mutant proteins validate the conclusions regarding the phosphorylation behavior of these mutants.

The method used by us for the localization of the phosphorylated residue on BglG can be applied to map phosphorylation sites on other proteins. Insertion mutagenesis, to introduce a protease cleavage site, or base substitutions, to change residues which are candidates for phosphorylation sites, can be accomplished quickly and efficiently by polymerase chain reaction nowadays. In case the protein of interest is available as a purified polypeptide, mutagenesis of individual suspected residues is not required. Rather, the introduction of a proteolytic site into the protein, which enables sequencing by Edman degradation from internal positions (31), should enable the identification of the phosphorylated residue.

The bgl system represents a new family of bacterial regulatory systems involved in signal transduction (1). Transduction of a signal by this system involves reversible phosphorylation of the BglG response regulator on a histidine residue by the BglF membrane-bound sensor (3, 4).3 Other systems in various organisms were suggested to affiliate to this new family (11–19). The phosphorylation events involved in signal transmission by these systems are not well defined yet. Mapping of the phosphorylation site in BglG is an important step toward the definition and the characterization of the functional domains involved in the communication between sensors and regulators of this novel family of sensory systems. Comparison of the sequence around the BglG phosphorylation site with the respective sequences of its known homologues, e.g., SacY, SacT, and LicT in Bacillus subtilis and BglR in Lactobacillus lactis, reveals that the phosphorylated histidine in BglG, His-208, and the residues flanking it are conserved in all these proteins. It is worth mentioning that the other two histidines that we mutated in BglG, His-219 and His-278, are not conserved in these homologues.

The bgl system was the first example of a reversible phosphorylation modulating a specific and well characterized conformational change in a transcription factor, in this case a change in the oligomeric state of BglG which controls its activity. Whereas non-phosphorylated BglG is a dimer that can bind RNA and antiterminate transcription, phosphorylated BglG is an inactive monomer (6). Mapping and characterizing the nature of both the dimerization and the phosphorylation sites on BglG is important for the elucidation of the mechanism by which phosphorylation affects the dimeric state of a protein. Studies of the BglG dimerization domain are underway.

Mutant derivatives of BglG have been described that do not respond to negative regulation by BglF. These mutants, BglG33 and BglG4, led to constitutive expression of the bgl operon in vivo (20, 32) and showed little or no phosphorylation by BglF in vitro (3). The mutations have been shown to map to Aasp100 and His160, respectively.3 The identification of histidine 208 as BglG phosphorylation site suggests that these mutations are in other sites involved in BglG-negative regulation and phosphorylation. Whether they are part of the site which is recognized by the kinase and interacts with it or they are part of a phosphorylation module composed of residues remote from each other in the primary sequence but brought to close vicinity during folding awaits future structural studies.

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