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Multiple Phosphorylation Events Regulate the Activity of the Mannitol Transcriptional Regulator MtlR of the *Bacillus stearothermophilus* Phosphoenolpyruvate-dependent Mannitol Phosphotransferase System*

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D-Mannitol is taken up by *Bacillus stearothermophilus* and phosphorylated via a phosphoenolpyruvate-dependent phosphotransferase system (PTS). Transcription of the genes involved in mannitol uptake in this bacterium is regulated by the transcriptional regulator MtlR, a DNA-binding protein whose affinity for DNA is controlled by phosphorylation by the PTS proteins HPr and IICB^{mtl}. The mutational and biochemical studies presented in this report reveal that two domains of MtlR, PTS regulation domain (PRD)-I and PRD-II, are phosphorylated by HPr, whereas a third IIA-like domain is phosphorylated by IICB^{mtl}. An involvement of PRD-I and the IIA-like domain in a decrease in affinity of MtlR for DNA and of PRD-II in an increase in affinity is demonstrated by DNA footprint experiments using MtlR mutants. Since both PRD-I and PRD-II are phosphorylated by HPr, PRD-I needs to be dephosphorylated by IICB^{mtl} and mannitol to obtain maximal affinity for DNA. This implies that a phosphoryl group can be transferred from HPr to IICB^{mtl} via MtlR. Indeed, this transfer could be demonstrated by the phosphoenolpyruvate-dependent formation of [^{3}H]mannitol phosphate in the absence of IIA^{mtl}. Phosphoryl transfer experiments using MtlR mutants revealed that PRD-I and PRD-II are dephosphorylated via the IIA-like domain. Complementation experiments using two mutants with no or low phosphoryl transfer activity showed that phosphoryl transfer between MtlR molecules is possible, indicating that MtlR-MtlR interactions take place. Phosphorylation of the same site by HPr and dephosphorylation by IICB^{mtl} have not been described before; they could also play a role in other PRD-containing proteins.

Many bacteria transport D-mannitol and other carbohydrates via a phosphoenolpyruvate-dependent phosphotransferase system (PTS)[1] (1–3). Recently, the mannitol operon of *Bacillus stearothermophilus* was cloned (4) and shown to consist of four genes, *mtlA*, *mtlR*, *mtlF*, and *mtlD*, coding for the mannitol transporter IICB^{mtl}, the transcriptional regulator MtlR, the phosphotransferase IIA^{mtl}, and mannitol-1-phosphate dehydrogenase, respectively. Analysis of the mannitol promoter revealed a catabolite response element overlapping the mannitol promoter, indicating that this operon is sensitive to catabolite repression. When favorable catabolites like glucose are utilized, HPr is phosphorylated by a kinase on a specific serine (5) that forms a complex with the CcpA repressor. Binding of this complex to catabolite response element sites located in or near the promoter regions of catabolic operons will prevent expression of these operons (6). In addition to catabolite repression, the expression of the mannitol operon is probably also regulated by the mannitol regulator MtlR (7). Domains in this protein show similarity to domains of two types of transcriptional regulators: DNA-binding proteins and anti-terminators. A helix-turn-helix motif is situated at the N terminus that is similar to those of DNA-binding transcriptional regulators of the DeoR family. The center of the protein sequence contains two domains resembling the PTS regulation domains (PRD-I and PRD-II) of the anti-terminators LicT, SacY, and BglG (7, 8). Anti-terminators are RNA-binding proteins that prevent premature termination of transcription at a terminator located between the promoter and the functional genes. Combinations of DNA-binding helix-turn-helix motifs and PRDs have been found in other proteins such as LevR and LicR. The activity of most of these proteins can be regulated by phosphorylation of PRD-I and/or PRD-II by the PTS components HPr and/or IIB. Based on these similarities, it was assumed that MtlR is a DNA-binding protein whose activity is regulated by the PTS (8).

In this paper, we link phosphorylation of the individual domains of MtlR by HPr and IICB^{mtl} to the regulation of this protein. The residues involved in HPr- and IICB^{mtl}-dependent phosphorylation are mapped and correlated with either an increase or decrease in the affinity of MtlR for DNA. In addition to PRD-I and PRD-II, a third phosphorylation domain is presented that is involved in the phosphorylation and dephosphorylation of MtlR by IICB^{mtl}.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, *Taq* DNA polymerase, nucleotides, oligonucleotide kinase, pyruvate kinase, and isopropyl-1-thio-β-D-galactopyranoside were purchased from Roche Molecular Biochemicals. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]mannitol (15–30 Ci/mmol) were obtained from Amersham Pharmacia Biotech and ICN, respectively. RNase-free RQ1 DNase I was obtained with the Promega Core footprinting system, and Ni^{2+}-nitrilotriacetic acid-agarose was from QIAGEN Inc. Primers were synthesized by Eurosequence B. V. Groningen. P-enolpyruvate and yeast tRNA were purchased from Sigma. Site-directed mutagenesis was performed with the QuickChange kit from Stratagene. α-Chymotrypsin (50.5 units/mg) was obtained from Worth-

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[1] The abbreviations used are: PTS, phosphoenolpyruvate-dependent phosphotransferase system; IICB^{mtl}, mannitol permease; IIA^{mtl}, enzyme IIA of the mannitol PTS; HPr, histidine phosphocarrier protein; EI, enzyme I of the PTS; PRD, PTS regulation domain; P-enolpyruvate, phosphoenolpyruvate; PCR, polymerase chain reaction; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

ington. Anti-His tag and anti-mouse antibodies were purchased from Amersham Pharmacia Biotech and Sigma, respectively.

Purification of B. stearothermophilus EI, HPr, IIA^{mtl}, IICB^{mtl}, and MtlR—MtlR and mutants of MtlR were overexpressed in *Escherichia coli* BL21(DE3) (9); *B. stearothermophilus* EI and HPr were expressed in *E. coli* ZSC112LΔHIC (10); and *B. stearothermophilus* IIA^{mtl} was expressed in *E. coli* JM101 (11). These proteins were purified as described by Henstra *et al.* (7). The *B. stearothermophilus* mannitol transporter IICB^{mtl} was expressed in the mannitol deletion *E. coli* strain LGS322 (12) and purified as described by Henstra *et al.* (4). PTS protein activities were measured as mannitol phosphorylation activity as described by Robillard and Blaauw (13).

General Methods—DNA was isolated from agarose gels using the gel extraction kit from QIAGEN Inc. Protein concentrations were determined according to Bradford (14). General DNA manipulations were performed as described by Sambrook *et al.* (15). Sequence data base searches were performed using the program BLAST at NCBI (16).

MtlR Mutants—The mutants of MtlR that were made following the QuickChange kit protocol of Stratagene are listed in Table I. Two complementary primers containing the mutation were created and used in a PCR amplifying 25 ng of the MtlR expression plasmid pETMtlRhis. The sequences of one strand of each of the complementary primers are listed in Table II. The PCR mixture was first incubated for 10 min at 94 °C, followed by 18 cycles of 1-min denaturation at 94 °C, 1-min annealing at 52 °C, and 16-min extension at 68 °C. Methylated template DNA was digested by *Dpn*I, and the remaining PCR product was precipitated and re-dissolved in 2 μl of triple-distilled water. After transformation to *E. coli* XL1-Blue, the plasmid was isolated and checked for the mutation by restriction analysis. After checking the entire MtlR sequence of a mutant, the plasmid was transformed to the T7 expression strain BL21(DE3). Double mutants of MtlR were created in a second round using one of the single mutants as template exactly as described above.

DNA Footprinting—DNA footprinting was performed essentially as described by Henstra *et al.* (7). A single-end ^{32}P-labeled DNA probe of the mannitol promoter region was synthesized in a PCR in which one of the primers was labeled. 28.5 pmol of the forward primer sah1 (5′-GGC AGG TGA ATT GTT AAA G-3′, priming at positions −127 to −109) was labeled with 100 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) by T4 polynucleotide kinase as recommended by Roche Molecular Biochemicals. The labeled primer was purified by chloroform/phenol and chloroform extractions followed by ethanol precipitation. 19 pmol of the labeled forward primer was built into a 473-base pair probe by PCR in a mixture containing 10 mM Tris, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs, 2.5 units of *Taq* DNA polymerase, 57 pmol of universal reverse primer (5′-ACAGGAAA-CAGCTATGACC-3′), and 1 ng of template DNA. The pSK-derived subclone pSKCH550, containing the area of the mannitol promoter from *Cla*I (position −354) to *Hin*dIII (position +212), was used as template DNA. After 30 cycles of 1-min denaturation at 94 °C, 1-min annealing at 55 °C, and 1-min elongation at 72 °C, the 473-base pair PCR product was separated by electrophoresis on a 0.8% agarose gel and isolated from the gel with the QIAGEN gel extraction kit.

Before use, MtlR and its mutants were dephosphorylated by incubation at 30 °C for 2 h at pH 6.5. The protein was diluted to 1 μM in phosphorylation buffer (25 mM Tris (pH 7.5), 5 mM dithiothreitol, 5 mM $MgCl_2$, and 0.25% decylpolyethylene glycol, end concentration) containing different combinations of PTS components. The concentrations of these components, when added, were 5 mM P-enolpyruvate, 0.04 mg/ml EI, 9 μM HPr, 0.2 μM IIA, 0.02 μM IICB^{mtl}, and 5 mM mannitol. After 2 h of phosphorylation at 30 °C, the incubated protein was diluted to various concentrations in the same phosphorylation mixture without MtlR. A 30-μl volume of diluted MtlR was mixed with 20 μl of the DNA binding mixture (25 mM Tris (pH 8), 10 mM $MgCl_2$, 25% glycerol, 100 mM KCl, and 30 kcpm labeled DNA) and was incubated for 16 min at 30 °C. The DNA was digested by adding 50 μl of 10 mM Tris (pH 8.0), 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.03 units/μl RQ1 DNase I and incubated for 2 min at room temperature. The digestion was stopped with 90 μl of 0.6 M NaAc, 30 mM EDTA, 0.5% SDS, and 30 μg/μl yeast tRNA. The digest was then purified by chloroform/phenol extraction and precipitated with 2.5 volumes of EtOH at −20 °C. Samples were separated on a 5% sequencing gel.

Phosphorylation of MtlR—[^{32}P]P-enolpyruvate was synthesized following the method of Roossien *et al.* (17). MtlR and the PTS enzymes were diluted in phosphorylation buffer (25 mM Tris-HCl (pH 7.5), 0.5 mM $MgCl_2$, and 5 mM dithiothreitol). Following separation of the proteins on a 15% SDS-polyacrylamide gel by electrophoresis, phosphorylation of proteins was visualized with a Molecular Dynamics PhosphorImager 425. The autoradiogram was analyzed using the ImageQuant program.

Limited Proteolysis of MtlR—MtlR was phosphorylated by PTS enzymes as described above for 40 min at 30 °C. 1.7 M urea, 2.2 mM $CaCl_2$, and 30 μg/ml α-chymotrypsin were added to a 30 μg/ml concentration of the phosphorylated protein. After 2 min at 37 °C, sample buffer (4% SDS, 12% (w/v) glycerol, 50 mM Tris-HCl (pH 6.8), 2% (v/v) mercaptoethanol, and 0.01% Serva blue G) was added and quickly frozen in liquid nitrogen. Protein fragments were separated by Tricine/SDS-polyacrylamide gel electrophoresis according to Schagger and von Jagow (18) and transferred to a nitrocellulose membrane. Phosphorylated peptides were analyzed with the PhosphorImager; N-terminal His-tagged peptides were analyzed by Western blotting using anti-His tag antibodies.

TABLE I
List of MtlR mutants used

Plasmid	Mutant
pETMtlRhis	Wild-type His-tagged MtlR
pETMtlR-AHHH	H236A
pETMtlR-HAHH	H295A
pETMtlR-HHAH	H348A
pETMtlR-HHHA	H405A
pETMtlR-AAHH	H236A/H295A
pETMtlR-HHAA	H348A/H405A
pETMtlR-AHAH	H236A/H348A
pETMtlR-HAHA	H236A/H348A
pETMtlR-HHHH-H598A	H598A
pETMtlR-AHHH-H598A	H236A/H598A
pETMtlR-HAHH-H598A	H295A/H598A

RESULTS

Two domains, PRD-I and PRD-II, are expected to contain the phosphorylation sites based on the homology of MtlR to antiterminators such as SacY and BglG and the DNA-binding regulators LicR and LevR. Alignment of these domains reveals two conserved histidines in each PRD (Fig. 1, *B* and *C*) that could be the phosphorylation sites involved in the regulation of MtlR. To demonstrate their involvement, mutants were made in which one or two of the conserved histidines were replaced by alanine. Wild-type MtlR and mutant MtlR were expressed in *E. coli* BL21(DE3) and purified by Ni^{2+}-nitrilotriacetic acid-agarose chromatography. The expression levels, yield, and purity of all the mutants are comparable to those of the wild-type protein (data not shown). The purified proteins were used in [^{32}P]P-enolpyruvate-dependent phosphorylation and footprint experiments to examine the effects of the mutation on HPr- and IICB^{mtl}-dependent phosphorylation and the binding properties of the protein for the mannitol operator.

TABLE II
Sequences of one strand of each of the complementary primers used to create His-to-Ala mutants of MtlR

Bases changed are indicated in boldface; the codon of the alanine is underlined; and the position of the restriction site that was created or removed is in italics.

Mutation	Primer	Restriction site
H236A	3′-CGTATATTGCGTTAGTGGTC**GC*G***CT***AGC***CTTAGCAATAGAGCG-5′	*Nhe*1
H295A	3′-GGAGATCGGGTATATTA*C**C**ATG***GC***T**TTAATGGGAGCGAAACTTCG-5′	*Nco*1
H348A	3′-CGAAGATTTGGTCGTT**GCA***TTAAA***GCCAGCACTTTATCGCATCC-5′	Δ*Dra*1
H405A	3′-CGGTTATTTAGTTCTC**GC**CTTTG*C**T**GCAG***CATTATTAAGGG-5′	*Pst*1
H598A	3′-CGGAAACGTC*GCT**A**GC***TTTGTAC**GC**TACTCGAAGCCCTGC-5′	*Nhe*1

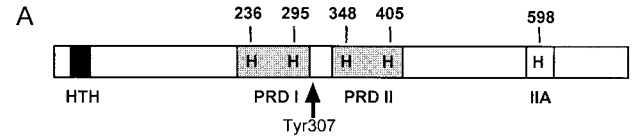

FIG. 1. **Localization and alignment of the putative phosphorylation sites of MtlR with those of other PRD-containing proteins and IIA proteins of the fructose family.** A schematic presentation of the locations of PRD-I, PRD-II, and the IIA-like domain in the sequence of MtlR is shown in *A*. The location of the α-chymotrypsin cleavage site (Tyr-307) of the experiment described in the legend of Fig. 3 is indicated by the *arrow*. The regions containing the putative phosphorylation sites of PRD-I (*B*), PRD-II (*C*), and the IIA-like domain (*D*) of MtlR are aligned with other PRD-containing proteins and IIA components of the fructose family of the PTS. Sequences used in the alignments are MtlR and IIA^{mtl} of *B. stearothermophilus* (*bst*, *b.ste*); SacT, SacY, LicT, LicR, LevR, MtlR, and $IIABC^{fru}$ of *B. subtilis* (*bsu*, *b.sub*); and BglG, IIA^{fru}, IIA^{ntr}, and IIA^{mtl} of *E. coli* (*eco*, *E.col*). HTH, helix-turn-helix.

B, PRD-I

```
                          236                                                  295
                           ↓                                                    ↓
MtlR-bst: 227 DSS IA  V  AL  E  SQ ESINFDQQYLETIQTT  YETAEKIARSLEH---AFRITI KE I   TM LMG KLRDR 304
SacT-bsu:  88 DHIHIA SD  S   E  QN LLVQNKLLHEIKALYK  YEIGLWAIGHVKE---TLGVS  E  A      HT KMDAE 165
SacY-bsu:  90 ERIHVAFSD  S   E  SN MVIKNPLLNEIKVLYP  FQIGLWARALIKD---KLGIH  D  I N  M  HT RNNAG 167
BglG-eco:  92 DSI IS TD CQ   K FQQNVLLPNPLLWDIQRLYP  FQLGEEALTIIDK---RLGVQ  K  V F  M  VS QMSGN 169
LicT-bsu:  91 DSI VS TD  N   Q NQK LDIKNALLWETKRLYKD FAIGKEALVMVKN---KTGVS  E  A F      VN ELNEE 168
LicR-bsu: 210 NMGLNN II  AI CK  RTENYVSLFPKDMDHILHQ  YQAAEAIVKELES---KLSVTF K  TA  TM  LGTKRMTQ 287
LevR-bsu: 497 RKFIYF SM  DAF K GKQIDVLNTQETDEIRDTHV  YRVAMIFKDKIQE---YFKVA  EI VI  TML HSIKSLKE 574
```

C, PRD-II

```
                          348                                                  405
                           ↓                                                    ↓
MtlR-bst: 339 YTL ED  V  KP  Y  QHNMGIANPLLEKIVQDYPELFAVLEKGVKQVF-----PDVTV K  I   V  FAA LLREK 414
SacT-bsu: 196 SIS QR  T  RY VS  ESNEALHRMDEEMLYFIQKKYSFAYQCALELAEFLKNEYQLH  ES A   T  VQRLQDLSE 276
SacY-bsu: 199 TIS ER  T  R  IQHIKAGESIYELDAEMIDIIKEKFKDAFLCALSIGTFVKKEYGFEF EK LC  AM  QRFYQRSV 278
BglG-eco: 199 SLS QR  T  K LSW  LEHASINDSDESLQQAVKQNYPQAWQCAERIAIFIGLQYQRK SPA IMF   N ERVRKEH  278
LicT-bsu: 198 SLH YRF T  K FAQ  FN THMESQDDFLLDTVKEKYHRAYECTKKIQTYIEREYEHK TS  LL  T   ERVVKQA  277
LicR-bsu: 324 KELKIG AL MKP  S NRY MNLRNPMLAAIKEHYPLAFEAGIIAGIVIKE---QTGIE HEN I      FGA IERKK 401
LevR-bsu: 860 NAVLIKVIM TA  FE VIKQNPIAFLEEEEINDQLKEMVYVTERTLAPYEE---KLGLR SD  KLFI AIFAEEVHGQL 937
```

D, IIA like domain

```
                                                598
                                                 ↓
MtlR b.ste      IIRNSEKVLTE  R  TL-GL  PETS    Y T SPAVVFP 607
IIABC^FRU B.sub KLNDRQAYKEA  N  SQSS   - EG     A TASVINP  76
IIA^FRU E.col   FITDSHQFLQT  L  KMHS  F- SGV V  G SACVKQP  74
IIA^NTR E.col   LSLPPQVVFEA  T  KMGS   - NG     G LEEDTLR  82
IIA^MTL E.col   GGYVEPEYVQAM D  KLTP Y - ES  V  GTVEAKDRV  74
IIA^MTL B.ste   NNGYVEDSYIDKMFE ALTS YM- NF     GTEDAKQFV  71
LicR b.ste      ECGADEEIIDS FE  DMSP CF- NLV    PLVPQTKTT 568
MtlR b.sub      IRDPEKAA-VC AE  KQGGL  P TNM  Y L NDEIVLP 607
LevR B.sub      SLSNGLNE-AN  V  LCEDSLKKYLVFLN  HVIDMLLWE 847
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[^{32}P]P-enolpyruvate-dependent Phosphorylation—The removal of phosphorylation sites by mutating one or two of the conserved histidines was tested using [^{32}P]P-enolpyruvate as a substrate. MtlR and MtlR mutants were incubated in two different phosphorylation mixtures: one containing [^{32}P]P-enolpyruvate, EI, and HPr and the other containing [^{32}P]P-enolpyruvate, EI, HPr, IIA^{mtl}, and $IICB^{mtl}$. By using a relatively low HPr concentration in the second mixture, the observed phosphorylation could be attributed primarily to $IICB^{mtl}$-dependent phosphorylation; a control was performed with a mixture without $IICB^{mtl}$ to obtain HPr-dependent background phosphorylation levels under these conditions. The data in Fig. 2*A* show clearly that the replacement of each of the two conserved histidines of PRD-II prevented or strongly reduced the phosphorylation by HPr, whereas mutations in PRD-I did not noticeably affect HPr-dependent phosphorylation. This indicates that both histidines of PRD-II are essential for MtlR phosphorylation by HPr. It is possible that PRD-I is the phosphorylation target of $IICB^{mtl}$. However, all of the mutants with replacements in PRD-I or PRD-II could still be phosphorylated by $IICB^{mtl}$, including the H236A/H348A and H295A/H405A double mutants, in which a histidine is replaced in both PRD-I and PRD-II. This indicates that there is another or an additional phosphorylation target for $IICB^{mtl}$ on the protein.

A careful analysis of the MtlR sequence was performed to identify additional putative phosphorylation sites. A sequence with low sequence identity to IIA proteins of the fructose family, including IIA^{mtl} of *B. stearothermophilus*, was found at the C terminus of MtlR (Fig. 1*D*). IIA proteins or domains are responsible for the transfer of the phosphoryl group from HPr to the B domain of the transporter. The new putative phosphorylation site is His-598 since it aligns with the active-site phosphohistidines of the IIA proteins. To test whether His-598 is involved in PTS-dependent phosphorylation, H598A mutants were made, and ^{32}P-enolpyruvate-dependent phosphorylation was performed as described for the PRD mutants. Based on these experiments (Fig. 2*B*), HPr-dependent phosphorylation was not affected by the H598A, H236A/H598A, or H295A/H598A mutation. On the other hand, the $IICB^{mtl}$-dependent phosphorylation levels of all H598A mutants were affected; they did not exceed the HPr background phosphorylation. These data indicate that the H598A mutation strongly reduces or completely inhibits $IICB^{mtl}$-dependent phosphorylation.

The phosphorylation experiments described above do not exclude HPr-dependent phosphorylation of PRD-I. We must consider whether mutations in PRD-II have reduced the efficiency of phosphorylation of PRD-I by HPr. Initial protease digestion experiments showed that MtlR can be primarily cut at sites in between PRD-I and PRD-II (data not shown). This implied that the phosphorylation of PRD-I by HPr or $IICB^{mtl}$ could be resolved using limited proteolysis of MtlR. This was done by phosphorylation of wild-type MtlR by HPr or $IICB^{mtl}$ using [^{32}P]P-enolpyruvate, followed by partial digestion of the phosphorylated protein by α-chymotrypsin. Phosphorylated fragments were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The phosphorylated fragments were visualized using a PhosphorImager (Fig. 3*A*). Fragments containing the N-terminal His tag were colored using His tag-specific antibodies (Fig. 3*B*). The mass of each fragment was determined using a partial CNBr digest of MtlR as a reference.

Digestion of the intact protein, phosphorylated by HPr (*Band I* in Fig. 3*A*, *lane 1*) and digested by α-chymotrypsin, resulted in two new labeled fragments with masses of 44 and 36 kDa (*Bands II* and *III*, respectively, in Fig. 3*A*, *lane 2*). The 36-kDa band was identified as an N-terminal fragment by the anti-His tag antibodies (Fig. 3*B*, *lane 2*). Both bands are probably the result of a single cut of MtlR at tyrosine 329 (tyrosine 307 in the non-His-tagged protein) since the masses of both bands add up to that of the intact protein. This implies that HPr can phosphorylate Band III containing PRD-I and Band II containing PRD-II and the IIA-like domain. Both fragments were phos-

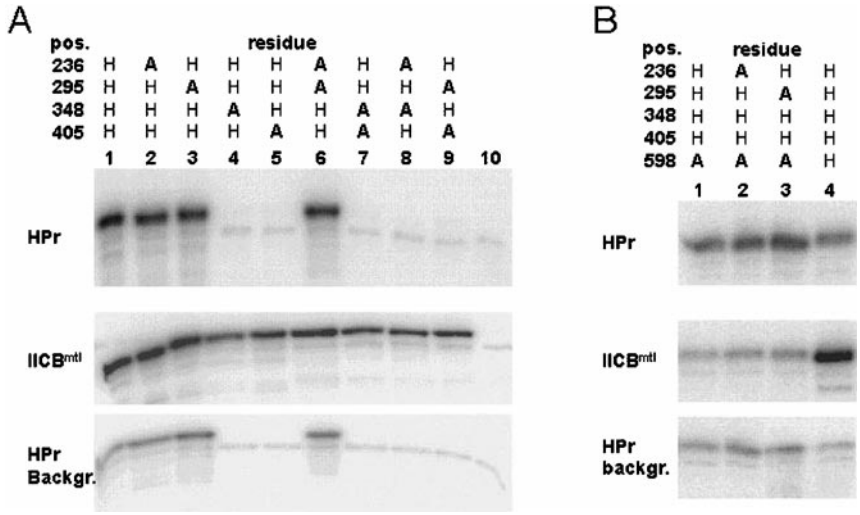

FIG. 2. **Phosphorylation of MtlR and MtlR mutants by HPr and IICB^{mtl}.** Shown is the phosphorylation of MtlR and MtlR mutants with single or double mutations of putative histidine phosphorylation sites replaced by alanine in PRD-I and PRD-II (*A*) and the IIA-like domain (*B*). Replacement of histidine by alanine is indicated by ***A*** above the lanes. The positions (*pos.*) of these residues are indicated to the left. The control experiment without MtlR is presented in *lane 10*. In the *upper panels*, phosphorylation was carried out with 8 μM [^{32}P]P-enolpyruvate, 0.04 mg/ml EI, and 5 μM HPr. In the *middle panels*, phosphorylation was carried out with 8 μM [^{32}P]P-enolpyruvate, 0.04 mg/ml EI, 0.5 μM HPr, 0.4 μM IIA^{mtl}, and 0.02 μM IICB^{mtl}. In the *lower panels*, a reaction without IICB^{mtl} was performed for each mutant to determine the background of HPr-dependent phosphorylation (*HPr Backgr.*) in the IICB^{mtl} phosphorylation experiment. The mixtures were incubated for 5 min at 30 °C, and the reactions were then started by the addition of 0.09 mg/ml MtlR or MtlR mutant. After 20 min at 30 °C, the reactions were stopped with 0.4 volume of denaturation buffer.

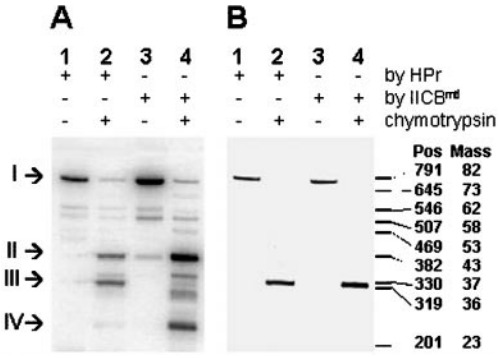

FIG. 3. **Limited proteolysis of MtlR phosphorylated by HPr and IICB^{mtl}.** MtlR was phosphorylated by HPr (*lanes 1* and *2*) and IICB^{mtl} (*lanes 3* and *4*) exactly as described for Fig. 2*A*. Part of the phosphorylated MtlR protein was digested by α-chymotrypsin for 2 min. Both uncleaved MtlR (*lanes 1* and *3*) and cleaved MtlR (*lanes 2* and *4*) were analyzed by SDS-polyacrylamide gel electrophoresis as described under "Experimental Procedures." The autoradiogram presenting phosphorylated peptides is shown in *A*, and the Western blot showing only the N-terminal His-tagged peptides is shown in *B*. *Band I* is uncleaved MtlR, and *Bands II* and *III* are cleavage products of interest. His-tagged MtlR, partly cleaved by CNBr, was used as a reference. The positions (*Pos*) of the N-terminal His-tagged peptides in the CNBr digest are indicated to the right. For each CNBr cleavage fragment, the cleavage location in the sequence and the mass of the peptide are given.

phorylated to approximately the same extent. When the protein was phosphorylated by IICB^{mtl}, only Band II, containing PRD-II and the IIA-like domain, was phosphorylated. This excludes a possible phosphorylation of PRD-I by IICB^{mtl}.

The weak bands, visible in lanes containing the uncut protein (Fig. 3), are probably phosphorylated degradation products of MtlR that could not be removed during purification (7). Additional cleavage products containing the IICB^{mtl} phosphorylation site His-598 could explain the additional labeled polypeptides such as fragment IV present only in digests of MtlR phosphorylated by IICB^{mtl} (Fig. 3*A*, *lane 4*).

Phosphorylation and the Affinity of MtlR Mutants for DNA Binding—The affinity of MtlR for DNA is dependent on its phosphorylation state (7). Since mutations affecting PTS-dependent phosphorylation would also affect the response of the protein to different phosphorylation conditions, we performed quantitative DNA footprint experiments using several mutants. Four different phosphorylation conditions per mutant were examined: 1) dephosphorylation of MtlR in the presence of EI, HPr, IIA^{mtl}, IICB^{mtl}, and mannitol (Fig. 4, □); 2) phosphorylation of MtlR by HPr in the presence of P-enolpyruvate, EI, and HPr (○); 3) phosphorylation of MtlR by HPr and IICB^{mtl} in the presence of P-enolpyruvate, EI, HPr, IIA^{mtl}, and IICB^{mtl} (△); and 4) phosphorylation of MtlR by HPr in the presence of P-enolpyruvate, EI, and HPr and simultaneous dephosphorylation by IICB^{mtl} and mannitol (▽). Since MtlR was purified in a phosphorylated form, MtlR and its mutants were first dephosphorylated by incubation at pH 6.5 for 2 h at 30 °C. After incubation of MtlR and the mutant proteins under the different phosphorylation conditions, the proteins were diluted to various concentrations using the same phosphorylation mixtures to maintain identical phosphorylation conditions during the DNA binding experiments for all dilutions. Binding of MtlR to DNA was followed by measuring the intensity of the footprint located at positions −46 to −41 (7). The level of protection of this region at each protein concentration was calculated from the decrease in intensity of this area compared with that of the unprotected DNA. These values are plotted against the logarithm of the protein concentration for each phosphorylation condition and each mutant in Fig. 4. The midpoint of the sigmoidal curves represents the concentration of MtlR that gives a protection level of 50% and is a measure of the affinity of the protein for DNA. Wild-type MtlR (Fig. 4*A*) behaved under the four phosphorylation conditions as observed previously (7). Phosphorylation by HPr resulted in a small increase, whereas phosphorylation by HPr and IICB^{mtl} resulted in a decrease in binding affinity compared with the non-phosphorylated protein. Maximal stimulation was observed with the combination of phosphorylation of MtlR by HPr and dephosphorylation via IICB^{mtl} and mannitol.

When one of the conserved histidines in PRD-I was replaced (Fig. 4, *B* and *C*), the affinity of these mutants, when phosphorylated by HPr (○) alone or by HPr and IICB^{mtl} (△), increased 4–5-fold compared with the affinity of wild-type MtlR under

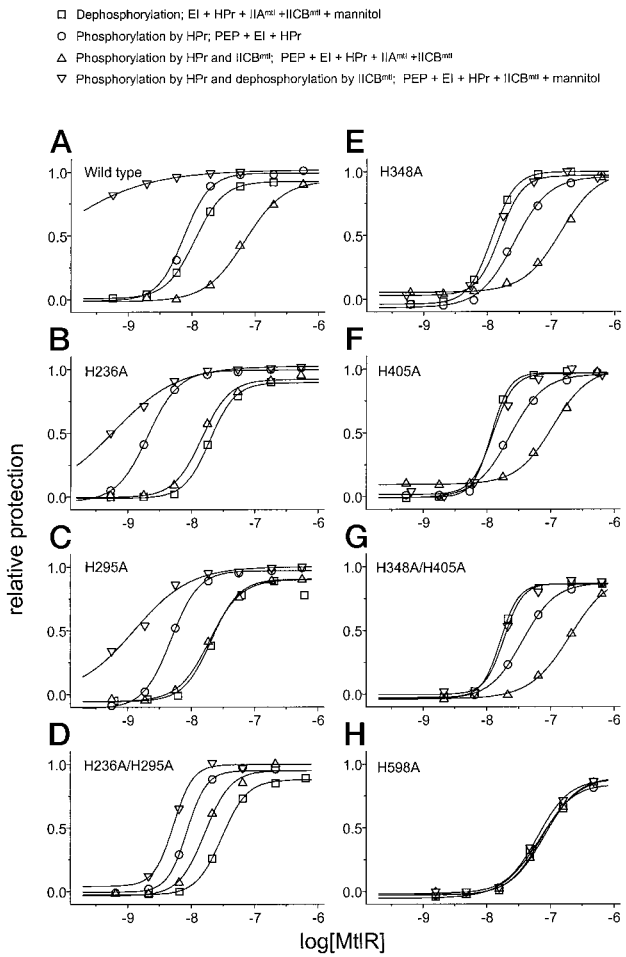

FIG. 4. **DNA binding of MtlR and MtlR mutants exposed to different phosphorylation conditions.** MtlR and MtlR mutants were dephosphorylated or phosphorylated by adding different components of the PTS, including P-enolpyruvate and mannitol, as described under "Results." After incubation, the samples were diluted to different concentrations of MtlR, and the binding to DNA was determined by DNA footprinting as described under "Experimental Procedures." For each phosphorylation condition, the relative protection is plotted against the logarithm of the concentration of MtlR or MtlR mutant. The relative protection is a measure of the number of DNA molecules bound by MtlR under the applied conditions. The four phosphorylation conditions and the components present in each of these conditions are presented at the top. The concentrations of these components, when added, were as follows: 5 mM P-enolpyruvate, 0.04 mg/ml EI, 9 μM HPr, 0.2 μM IIA^{mtl}, 0.02 μM IICB^{mtl}, and 5 mM mannitol. The mutant used is indicated in each panel.

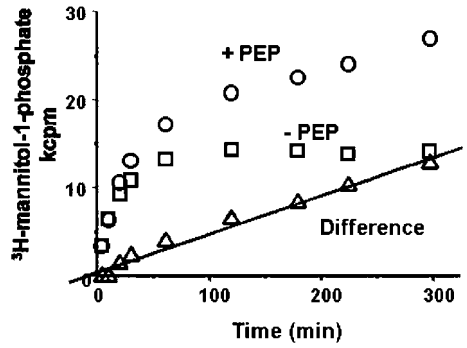

FIG. 5. **[^{3}H]Mannitol phosphorylation catalyzed by MtlR and components of the PTS.** The formation of [^{3}H]mannitol phosphate by 0.04 mg/ml EI, 1.5 μM HPr, 0.05 mg/ml MtlR, and 0.02 μM IICB^{mtl} was followed in the presence (○) and absence (□) of P-enolpyruvate (*PEP*). The P-enolpyruvate-dependent component of the [^{3}H]mannitol phosphorylation (△) was calculated from the difference between the reaction with and without P-enolpyruvate. The slope of the P-enolpyruvate-dependent reaction was determined by linear regression and is a measure for the phosphoryl transfer rate from P-enolpyruvate to [^{3}H]mannitol via the PTS.

these conditions (Fig. 4*A*). This indicates that PRD-I is probably involved in the negative control of MtlR by HPr and IICB^{mtl}. An additional site involved in negative control is likely since the affinity of these mutants phosphorylated by HPr alone could be reduced by the addition of IIA^{mtl} and IICB^{mtl}. Also, the affinity of PRD-I mutants phosphorylated by HPr could still be increased when they were simultaneously dephosphorylated by IICB^{mtl} and mannitol (▽), but to a lesser extent than wild-type MtlR. The affinity of the PRD-I double mutant H236A/H295A (Fig. 4*D*) was reduced compared with those of the single mutants. The trends observed for the single mutants were still present in the double mutant, except when the protein was phosphorylated by HPr and IICB^{mtl} (△). Under these phosphorylation conditions, stimulation of affinity was observed compared with the non-phosphorylated mutant (□), which was not seen in the H236A or H295A single mutant (compare □ and △ in Fig. 4, *B–D*).

If one or both conserved histidines of PRD-II was replaced by alanine (Fig. 4, *E–G*), the positive effects of phosphorylation by HPr disappeared (▽ and ○). Phosphorylation by HPr alone (○) resulted even in a decrease in affinity for the DNA compared with the non-phosphorylated protein (□). The negative effects of phosphorylation by IICB^{mtl} appeared to be unaffected in these mutants (△). The observed negative effect of phosphorylation by HPr (○) on these mutants disappeared if the protein was simultaneously dephosphorylated by IICB^{mtl} and mannitol (▽). However, under these conditions, the affinity of the protein did not exceed that of the non-phosphorylated protein (compare ▽ and □). Replacement of His-598 by alanine had a dramatic effect on the regulation of MtlR by the PTS. The H598A mutant had a low affinity under all phosphorylation conditions, comparable to that of wild-type MtlR phosphorylated by HPr and IICB^{mtl}.

MtlR-dependent Phosphoryl Transfer from HPr to IICB^{mtl}— It was suggested that HPr could play a dual role in the regulation of MtlR since maximal stimulation of DNA binding was observed when MtlR was both phosphorylated by HPr and dephosphorylated by IICB^{mtl}. Dephosphorylation of one of the PRDs by IICB^{mtl} could be a possible explanation. When a site can be phosphorylated by HPr and subsequently dephosphorylated by IICB^{mtl}, P-enolpyruvate-dependent phosphoryl transfer from HPr to IICB^{mtl} via this site on MtlR must be possible. The transfer of a phosphate group from P-enolpyruvate via EI, HPr, MtlR, and IICB^{mtl} to mannitol can be followed by measuring the formation of [^{3}H]mannitol phosphate as described under "Experimental Procedures." The formation of mannitol phosphate was followed against time in the presence (Fig. 5, ○) and absence (□) of P-enolpyruvate. In the absence of P-enolpyruvate, formation of mannitol phosphate to a certain level was observed, indicating that one or several of the added proteins were already phosphorylated. The P-enolpyruvate-independent phosphorylation of mannitol was not observed if MtlR was replaced by IIA^{mtl}, indicating that MtlR is the phosphoryl donor in the P-enolpyruvate-independent phosphorylation event (Table III). The end level of the P-enolpyruvate-independent reaction is a measure for the number of phosphoryl groups present on MtlR. A linear increase in the level of mannitol phosphate was observed (△) when the difference between the reaction with (○) and without (□) P-enolpyruvate was plotted against the reaction time. The P-enolpyruvate-dependent phosphoryl transfer rate can be calculated from the slope of this line and is dependent on the MtlR concentration used (data not shown). The phosphoryl transfer via MtlR is not efficient

TABLE III
P-enolpyruvate-dependent and -independent phosphoryl transfer activities of MtlR and its mutants

The formation of [^{3}H]mannitol 1-phosphate as a measure of phosphoryl transfer by the PTS was followed against time as described in the legend of Fig. 5. The P-enolpyruvate-dependent phosphorylation rate was calculated from the slope of the difference between the reaction with and without P-enolpyruvate. The P-enolpyruvate-independent phosphorylation level is a measure of the number of phosphoryl groups present in the system, which was derived from the end level of [^{3}H]mannitol 1-phosphate formed in the absence of P-enolpyruvate. All reactions were carried out at comparable MtlR or MtlR mutant concentrations (~50 μg/ml). In the case of the H598A + H348A/H405A complementation experiment, both MtlR mutants were added to a concentration of 45 μg/ml. The activity in this case is expressed as the amount of [^{3}H]mannitol 1-phosphate formed per g of one of the mutants.

Mutant	PEP[a]-dependent activity	PEP-independent phosphorylation level
	nmol min^{-1} g^{-1}	*mol P/mol MtlR*
Wild-type	29	0.9
H236A	18	0.6
H295A	46	0.7
H236A/H295A	32	0.5
H348A	11	0.8
H405A	11	0.8
H348A/H405A	7.1	0.9
H236A/H348A	3.5	0.6
H295A/H405A	4.7	0.6
H508A	0.3	0.0
H598A + H348A/H405A	37	0.8
IIA^{mtl}	7600	0.0

[a] PEP, P-enolpyruvate.

compared with the phosphoryl transfer by IIA^{mtl}. The turnover via IIA^{mtl} is ~45 times higher than that via MtlR.

To assign domains in MtlR responsible for the observed phosphoryl transfer, P-enolpyruvate-dependent and -independent phosphorylation experiments were performed using the MtlR mutants. The plateau level of the P-enolpyruvate-independent reaction and the P-enolpyruvate-dependent phosphoryl transfer rate are listed in Table III. The phosphoryl transfer rate was decreased and increased by 50% for the PRD-I single mutants H236A and H295, respectively, whereas the activity was unaffected in the H236A/H295A double mutant. The H348A, H405A, and H348A/H405A histidine mutations in PRD-II resulted in decreased phosphoryl transfer activity to 36, 39, and 24%, respectively, compared with wild-type activity. Mutations in both PRD-I and PRD-II led to even further decreases in activity to 12 and 16% of wild-type activity for the H236A/H348A and H295A/H405A mutants, respectively. In the H598A mutant, phosphoryl transfer activity was completely absent, indicating that His-598 is essential for the observed phosphoryl transfer activity. In addition, the H598A mutant was the only mutant that had no P-enolpyruvate-independent phosphorylation activity. The protein is either not phosphorylated in *E. coli* or cannot be dephosphorylated by IICB^{mtl} and mannitol. For all other mutants and wild-type MtlR, comparable P-enolpyruvate-independent phosphoryl transfer levels were observed. Since the initial phosphorylation levels are not known, the number of phosphorylation sites per MtlR molecule cannot be determined with this method.

The *E. coli* β-glucoside regulator BglG has been observed as a monomer or a dimer, depending on its phosphorylation state (19). The formation of di- or multimers could also play a role in the regulation of the activity of MtlR. Complementation experiments were performed to test whether phosphoryl transfer from one MtlR molecule to another can take place in these putative MtlR multimers as described in the legend of Table III. In these experiments, we determined the P-enolpyruvate-dependent phosphoryl transfer rate of a mixture of the inactive H598A mutant and the PRD-II H348A/H405A double mutant with an activity of 24% compared with the wild-type protein. Combination of equal amounts of the PRD-II H348A/H405A mutant and the inactive H598A mutant resulted in the recovery of phosphorylation activity. 127% of wild-type activity was found when the activity was calculated as the phosphorylation rate/mg of one of the mutants (Table III). The double amount of

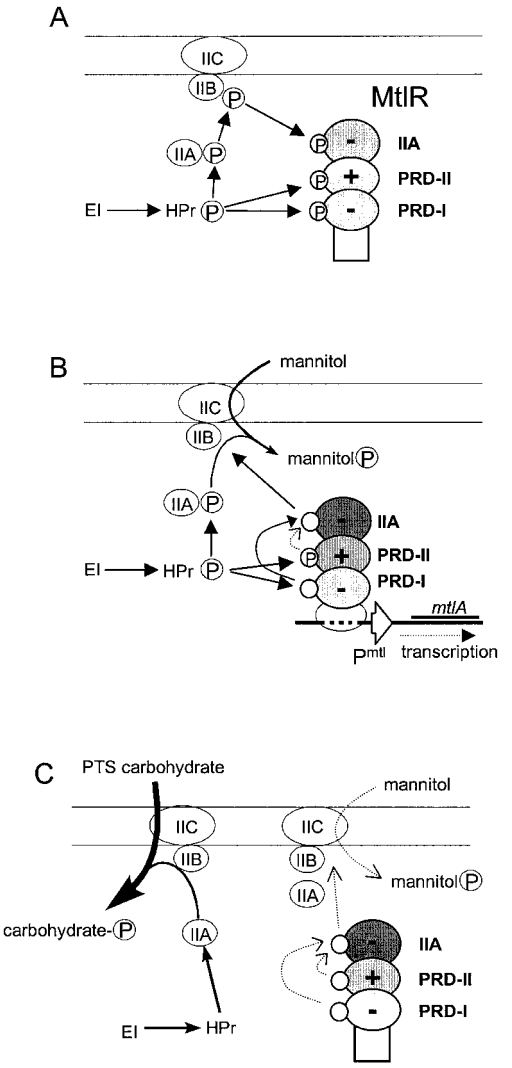

FIG. 6. **Proposed model for the regulation of MtlR by the PTS.** MtlR can be phosphorylated on several domains, leading to an increased (+) or decreased (−) affinity for the mannitol promoter region. Phosphorylation of PRD-II by HPr results in an increased affinity of MtlR for DNA, whereas phosphorylation of PRD-I by HPr and of the IIA-like domain by IICB^{mtl} prevents binding to this region. Stimulation by phosphorylated PRD-II is only possible when the domains involved in negative regulation, PRD-I and the IIA-like domain, are dephosphorylated. *A*, in the absence of mannitol and other substrates, all PTS proteins will be in the phosphorylated state. MtlR will be phosphorylated by HPr and IICB^{mtl} on all its domains and have a low affinity for the mannitol operator. Since MtlR is probably a transcriptional activator, the expression of the mannitol operon is not stimulated under these conditions. *B*, in the presence of mannitol, IICB^{mtl} is dephosphorylated by transfer of the phosphoryl group to mannitol. The IIA-like domain and PRD-I, which are involved in negative regulation, are dephosphorylated via IICB^{mtl}, and maximal P-HPr stimulation of MtlR by phosphorylation of PRD-II can take place. *C*, when rapidly metabolizable PTS substrates, including mannitol, are transported, the concentration phospho-HPr decreases. Consequently, MtlR is no longer phosphorylated at PRD-II, and the expression of the mannitol operon is no longer stimulated.

intact PRD-I domain in the complementation reaction compared with the reaction of wild-type MtlR could explain a complementation beyond 100% activity.

DISCUSSION

Interplay between HPr- and IICB^{mtl}-dependent Phosphorylation and Dephosphorylation in the Regulation of MtlR—MtlR senses the presence of mannitol and the need to utilize this substrate by monitoring the phosphorylation state of HPr and IICB^{mtl}. Depending on the amounts of HPr, phospho-HPr, IICB^{mtl}, and phospho-IICB^{mtl}, phosphorylation or dephosphorylation of the individual domains of MtlR in the cell will lead to the stimulation or reduction of the expression of the mannitol operon as shown in Fig. 6. The phosphorylation level of HPr is dependent on the rate of uptake of all PTS carbohydrates, whereas that of IICB^{mtl} is dependent only on the uptake rate of mannitol. At low PTS activities, phospho-HPr accumulates. MtlR is phosphorylated on both PRDs, resulting only in a slight stimulation of binding to DNA (Fig. 6*A*). Before full stimulation of MtlR by phosphorylated PRD-II can take place, PRD-I needs to be dephosphorylated by IICB^{mtl} and mannitol (Fig. 6*B*). In the absence of phospho-HPr, MtlR is not phosphorylated on PRD-II and will not be stimulated to bind to the mannitol promoter region (Fig. 6*C*).

Location of the HPr- and IICB^{mtl}-dependent Phosphorylation Sites in MtlR—Phosphorylation reactions with MtlR mutants indicated that both His-348 and His-405 of PRD-II are involved in the stable HPr-dependent phosphorylation. Whether PRD-I was phosphorylated by HPr could not be concluded from such experiments. Instead, α-chymotrypsin cleavage of wild-type MtlR at a location in between PRD-I and PRD-II was employed. It revealed that PRD-I was phosphorylated by HPr; however, the process was dependent on a functional PRD-II. Single mutations in PRD-II strongly reduced the total HPr-dependent phosphorylation of MtlR, whereas PRD-I and PRD-II were phosphorylated to the same order of magnitude when phosphorylation of the wild-type protein was studied in the α-chymotrypsin digestion experiments. In contrast, mutations in PRD-I seemed not to affect phosphorylation of PRD-II by HPr. A similar relation between a mutation in one PRD and the phosphorylation of another PRD has been observed for SacY and BglG (20–22). These observations suggest that phosphorylation of PRD-I and that of PRD-II are not independent reactions. Even the two phosphorylation sites within one PRD are not phosphorylated independently. In the case of PRD-II, a single mutation of either of the two phosphorylation sites results in the loss of HPr-dependent phosphorylation.

HPr-dependent Regulation of MtlR—The affinity of MtlR for its DNA-binding site is regulated by phosphorylation via HPr or IICB^{mtl}. Our previous work suggested that phosphorylation by HPr could have two effects, one leading to an increase and the other to a decrease in the affinity of MtlR for DNA (7). The current study shows that HPr phosphorylates both PRD-I and PRD-II. The increased affinity is probably due to phosphorylation of PRD-II because, when phosphorylation sites are removed from this domain, phosphorylation of MtlR by HPr no longer results in an increased affinity for DNA. Similarly, the decreased affinity is due to the phosphorylation of PRD-I; removal of phosphorylation sites from this domain results in a protein that, when phosphorylated by HPr, possesses much higher affinities than wild-type MtlR for DNA.

The relationship between positive and negative regulation and phosphorylation of PRD-II and PRD-I, respectively, correlates with that of the anti-terminator SacT and probably LicT. SacT is involved in the activation of the *sacPA* operon of *Bacillus subtilis*. Mutations in PRD-I of SacT result in the loss of negative control by the PTS upon expression of the *sacPA* operon (23, 24). The involvement of PRD-II in the positive regulation of SacT was suggested by site-directed mutagenesis studies.[2] For the anti-terminator LicT, the involvement of PRD-II with positive regulation has been confirmed (25). Whether PRD-I in this protein is responsible for the observed negative control by the PTS is still unclear (26–28). In contrast with LicT, SacT, and MtlR, phosphorylation of PRD-II in BglG and LevR is correlated with a negative regulation of these proteins (21). PRD-I is responsible for the positive regulation of LevR (29, 30).

IICB^{mtl}-dependent Regulation of MtlR—The affinity of wild-type MtlR is decreased by phosphorylation by IICB^{mtl}. Analysis of the chymotrypsin cleavage data indicated that PRD-II is phosphorylated in a IICB^{mtl}-dependent manner that is contingent on the presence of His-598 in the IIA-like domain. This same analysis showed that PRD-I is not phosphorylated by IICB^{mtl}. Nevertheless, the affinity of PRD-I mutants phosphorylated by HPr can be reduced by additional phosphorylation by IICB^{mtl}. This points to a negative control site outside of PRD-I that can be phosphorylated by IICB^{mtl}. The most likely candidate is His-598 in the IIA-like domain. Mutation of His-598 to alanine was expected to release the negative effect of phosphorylation by IICB^{mtl}, but instead resulted in low affinity under all phosphorylation conditions. The mutation of His-598 could influence the structure of MtlR, resulting in the low affinity of this mutant. The possible inability of this mutant to dephosphorylate the site involved in negative control, PRD-I, is a less likely explanation since the H236A/H598A double mutant gives a similar result compared with the H598A single mutant in DNA binding experiments (data not shown).

IICB^{mtl} is also needed for the release of negative control. Maximal HPr-dependent stimulation of MtlR-DNA binding is observed only in the presence of IICB^{mtl} and the substrate mannitol. Dephosphorylation of sites of MtlR involved in negative control such as PRD-I by IICB^{mtl} and mannitol could be an explanation. A relation between the response to an available substrate and the corresponding permease is also found for other PRD-containing proteins, as demonstrated for the combinations BglG/BglF, SacY/SacX, LicT/BglP, GlcT/IICBA^{glc}, and LevR/LevE (28, 31–34). Mutations affecting the phosphorylation of these permeases resulted in constitutive expression of the genes under control of the corresponding transcriptional activators. Whether a IICB^{mtl} mutation in *B. stearothermophilus* will lead to constitutive expression of the mannitol operon is questionable since the *in vitro* DNA binding of MtlR phosphorylated in the absence of IICB^{mtl} is only slightly stimulated by HPr compared with the non-phosphorylated protein.

Dephosphorylation of the PRDs by IICB^{mtl} via the IIA-like Domain in MtlR—The above-proposed dephosphorylation of PRD-I by IICB^{mtl} implies that phosphorylation sites on PRD-I are directly or indirectly accessible to both HPr and IICB^{mtl}. This is confirmed by the observed phosphoryl transfer from HPr to IICB^{mtl} via MtlR. Internal phosphoryl transfer from one site to the other within MtlR is likely since HPr and IICB^{mtl} have different phosphorylation targets on MtlR. Indeed, both the PRDs and His-598 appear to be involved in the phosphoryl transfer, as was demonstrated using MtlR mutants. His-598 is essential, indicating an important role for the IIA-like domain in this process. Mutations in PRD-I and PRD-II also affect phosphoryl transfer; however, phosphoryl transfer was not abolished for any of the PRD mutants, including the H236A/H295A and H348A/H405A double mutants, demonstrating that phosphoryl transfer is not solely dependent on one of the two PRDs. Probably both PRD-I and PRD-II can be dephospho-

[2] M. Arnaud, unpublished results.

rylated by $IICB^{mtl}$. Even PRD-I/PRD-II double mutants showed some phosphoryl transfer activity. At this point, direct phosphoryl transfer from HPr to the IIA-like domain cannot be excluded completely. Mutations in PRD-I and PRD-II could affect this process and explain the observed differences in the phosphoryl transfer rates of the various mutants.

An indication that phosphoryl transfer from the PRDs to the IIA-like domain takes place is the complementation observed when two mutant proteins, the PRD-II mutant H348A/H405A and the IIA-like domain mutant H598A, were combined. The low activity of the PRD-II double mutant could be restored by the inactive H598A mutant. This demonstrates that the phosphoryl groups can be transferred between MtlR molecules and could be seen as evidence for a functional interaction between two MtlR molecules with transfer occurring over the MtlR-MtlR interface. An increase in phosphoryl transfer activity was observed for the PRD-I mutant H295A compared with the wild-type protein, suggesting that the rate of transfer via PRD-II is affected by this mutation. Differences in the association state for the various MtlR combinations could be responsible since they would affect the proposed phosphoryl transfer from one MtlR molecule to the other. Changes in the association state upon phosphorylation could be the mechanism controlling the affinity of the protein for DNA.

Conclusion—MtlR and the PTS provide a regulatory system that can monitor the presence of the substrate and the need to utilize it. MtlR is the first protein in the class of PRD-containing transcriptional regulators for which a dual effect on the activity of MtlR by HPr-dependent phosphorylation has been shown. Also, the phosphorylation of one or more sites by HPr and the subsequent dephosphorylation of these sites via $IICB^{mtl}$ have not been described before. Whether other proteins in this class have similar properties is still unclear. For LicR and MtlR of *B. subtilis*, a similar mechanism can be expected since they are homologous to MtlR and contain two PRDs and a IIA-like domain as well. An interesting observation is the effect of PRD-II single mutations on the phosphorylation level of PRD-I. Mutations in PRD-II could influence the conformation of PRD-I and reduce its ability to be phosphorylated by HPr. However, the transfer of a phosphoryl group from PRD-II to PRD-I could be an alternative explanation. The phosphoryl transfer reaction between the PRDs and the IIA-like domain suggests a higher association state of MtlR that might be changed upon phosphorylation. The influence of phosphorylation on this association state will be the subject of further study.

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