High Affinity Binding and Allosteric Regulation of *Escherichia coli* Glycogen Phosphorylase by the Histidine Phosphocarrier Protein, HPr*

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The histidine phosphocarrier protein (HPr) is an essential element in sugar transport by the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Ligand fishing, using surface plasmon resonance, was used to show the binding of HPr to a nonphosphotransferase protein in extracts of *Escherichia coli*; the protein was subsequently identified as glycogen phosphorylase (GP). The high affinity (association constant ~10^9 M^-1), species-specific interaction was also demonstrated in electrophoretic mobility shift experiments by polyacrylamide gel electrophoresis. Equilibrium ultracentrifugation analysis indicates that HPr allosterically regulates the oligomeric state of glycogen phosphorylase. HPr binding increases GP activity to 250% of the level in control assays. Kinetic analysis of coupled enzyme assays shows that the binding of HPr to GP causes a decrease in the K_m for glycogen and an increase in the V_max for phosphate, indicating a mixed type activation. The stimulatory effect of *E. coli* HPr on *E. coli* GP activity is species-specific, and the unphosphorylated form of HPr activates GP more than does the phosphorylated form. Replacement of specific amino acids in HPr results in reduced GP activity; HPr residues Arg-17, Lys-24, Lys-27, Lys-40, Ser-46, Gln-51, and Lys-72 were established to be important. This novel mechanism for the regulation of GP provides the first evidence directly linking *E. coli* HPr to the regulation of carbohydrate metabolism.

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1 The abbreviations used are: PTS, phosphoenolpyruvate:sugar phosphotransferase system; SPR, surface plasmon resonance; RU, response units; EI, enzyme I of the PTS; IIA^Glc, enzyme IIA^Glc of the glucose PTS; IIA^Fru, enzyme IIA^Fru of the fructose PTS; NPr, the *E. coli* phosphocarrier protein that may control the state of phosphorylation of IIA^Fru; DTP, the diphosphoryl transfer protein of the *E. coli* PTS; GP, glycogen phosphorylase; HPr, histidine phosphocarrier protein; P-HPr, phosphorylated HPr; HPLC, high pressure liquid chromatography; RP-HPLC, reverse phase-HPLC; PAGE, polyacrylamide gel electrophoresis.

2 Further extensive information concerning surface plasmon resonance can be obtained on the World Wide Web at http://www.biacore.com.

This paper is available on line at http://www.jbc.org

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Before the present studies were carried out, there was no conclusive evidence that associated HPr with regulatory functions in Gram-negative bacteria, including E. coli; further, it was previously thought that E. coli and other prokaryotic GPs are not subject to regulation (16, 17).

EXPERIMENTAL PROCEDURES

Materials—Rabbit muscle glycogen phosphorylase a and b were from Sigma. Yeast glucose-6-phosphate dehydrogenase, yeast hexokinase, rabbit muscle phosphoglucosamutase, and restriction enzymes (Sall and Ndel were obtained from Boehringer Mannheim. Pf6 DNA polymerase was from Amersham. Mariner 12′24′ Wide-Range proteins extracted from were purchased from NOVEX. EII and IA′′′ from E. coli were purified after hyperexpression by a previously described method (18). HPr was expressed and purified using G16H8 transformed with expression vector pSP100 as described recently (19). HPrs from B. subtilis and Mycoplasma capricolum were gifts from Dr. Peng-Peng Zhu. A collection of HPrs carrying single replacements was a gift from Dr. E. Bruce Waygood. Dr. Jonathan Reizer generously provided essentially pure E. coli NPr as well as E. coli DTP (approximately 50% pure).

Protein Sequencing—Two samples of the HPr-binding protein (10 μg) were dried in a Speed Vac concentrator (Savant, Farmingdale, NY). The resulting residues were dissolved in 50 μl of 8 M urea, 0.4 M NH₄HCO₃, and subjected to reduction, alkylation, and proteolytic digestion with (a) 1.0 μg of endoprotease Lys-C (Boehringer Mannheim) or (b) 1.0 μg of modified trypsin (Promega, Madison, WI) according to the method of Stone and Williams (20). The resulting digests were separated by RP-HPLC on a narrow bore (2.1 × 250 mm) Vydac 218TP52 column/guard column combination (Separations Group, Hesperia, CA) with elution at 0.25 ml/min at 35 °C, utilizing the gradient described by Hernandez et al. (21) on a System Gold HPLC equipped with a model 507 autosampler, model 126 programmable solvent module, and model 168 diode array detector (Beckman, Fullerton, CA). Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. Column effluent was monitored at 215 and 280 nm. Fractions were collected at 30-s intervals and stored at –70 °C. Selected fractions from the trypsin digest containing mixtures of peptides were pooled, concentrated in a Speed Vac concentrator, and further purified by a second RP-HPLC step on a narrow bore (2.0 × 250 mm) YMC ODS AQ column (YMC, Morris Plains, NJ). Elution was at 0.25 ml/min at 35 °C on the same HPLC system utilizing the previously described gradient and buffers. Fractions (125 μl) containing peptides were applied in 30-μl aliquots to a Biobeads-treated glass fiber filter (Applied Biosystems, Foster City, CA) and dried prior to amino acid sequencing (Applied Biosystems), using methods and cycles supplied by the manufacturer. Data were collected and analyzed on a model 610A data analysis system (Applied Biosystems). Amino acid sequences were searched in the GCG-Swiss Protein Data base (University of Wisconsin Genetics Computer Group, Madison, WI).

Measurement of Protein-Protein Interaction—The interactions of glycogen phosphorylase according to analysis using SDS-PAGE (4–20% gradient gel, Novex). These fractions were pooled and concentrated in a 10 K Macrosep centrifugal concentrator (Filtron) in the presence of 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. At this stage, the GP was approximately 75% pure. The concentrated Mono-Q column and then stored at –20 °C.

Expression of Glycogen Phosphorylase—Cells grown overnight were used to inoculate 1 liter of synthetic medium (23) supplemented with 50 μg/ml ampicillin to an A₅₀₀ = 0.25–0.30, and the culture was incubated at 30 °C. When the culture reached an A₅₀₀ ~ 0.4, tryptophan (100 μg/ml) was added to induce glpP expression. The cells were harvested at an A₅₀₀ of 1.8 (7–8 h after induction) and washed once with 25 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, and the cell pellet was stored at –20 °C.

Purification of Glycogen Phosphorylase—The steps of purification were followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). GP activity was determined as described below and protein concentration was determined by the Bradford method (24) (see Table I). The cell pellet was resuspended in 25 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and then passed three times through a French press at 10,000 p.s.i. The lysate was cleared of cell debris by centrifugation at 17,000 × g for 100 min (crude extract). The majority of GP (approximately 80%) was found in the insoluble portion of the lysate due to inclusion body formation associated with the high level of GP expression. Approximately 15% of the protein in the crude extract corresponded to GP. The crude extract was chromatographed through an FPLC Mono-Q 10/10 column (Pharmacia) using a gradient of 100–500 mM NaCl (total volume, 80 ml). Fractions at approximately 450 mM NaCl were enriched in glycogen phosphorylase according to analysis using SDS-PAGE (4–20% gradient gel, Novex). These fractions were pooled and concentrated in a 10 K Macrosep centrifugal concentrator (Filtron) in the presence of 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. At this stage, the GP was approximately 75% pure. The concentrated Mono-Q fractions were chromatographed through a Superose 12 column (1.6 × 50 cm) using 25 mM Tris-HCl, pH 7.5, with 100 mM NaCl. The fractions were analyzed, pooled, and concentrated as described for the Mono-Q column and then stored at –80 °C. Table I shows the details of the purification. The overall purification was 7.7-fold from the crude extract to the final preparation. From 1 liter of culture (wet weight of cells, 3.24 g), a yield of 16.24 mg (24) of approximately 95% pure GP was obtained.

Glycogen Phosphorylase Activity Assay—Glycogen phosphorylase activity was measured using a phosphoglucomutase- and glucose-6-phosphate dehydrogenase-coupled enzyme assay (25). The reaction mixture (1 ml) contained 50 mM sodium phosphate, pH 7.4, 1.3 mM MgCl₂, 0.2% glycogen, 50 mM glucose-6-phosphate, 1 mM 2-mercaptoethanol, 0.6 mM NADP, 10 μg/ml phosphoglucomutase, 2 μg/ml glucose-6-phosphate dehydrogenase, and 5 μg/ml glycogen phosphorylase, unless noted otherwise. Two minutes after the initiation of the reaction with GP, the A₅₀₀, a measure of the increase in NADPH, was recorded every minute for a total of 10 min in a Pharmacia LKB Ultraspec Plus Spectropho-
A unit of glycogen phosphorylase activity is defined as the reduction of 1 μmol of NADP/min at 25 °C.

**Gel Shift Assay—PAGE** was used to demonstrate the shift of GP mobility due to its interaction with HPr in a non-denaturing discontinuous system (3% stacking gel in Tris·HCl, pH 6.7, and 6% resolving gel in Tris-glycine, pH 8.3). Trisglycine (pH 8.3) was used as the running buffer, and the temperature was maintained at 15 °C using a NOVEX ThermoFlow temperature controller. A binding mixture (10 μl) of 100 mM Tris·HCl, pH 7.5, 2 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 200 μg/ml glycogen phosphorylase, and 40 μg/ml of HPr (approximately a 2.1 molar ratio of HPr to GP) was allowed to incubate at room temperature for 10 min. This mixture was combined with 3 μl of 5 × loading buffer (0.01% bromphenol blue, 0.5 mM Tris·HCl, pH 6.8, 50% glycerol) and electrophoresed on a pre-equilibrated gel for 1 h at 70 V, followed by 2 h at 100 V. For analysis of gel shifts associated with mutant HPrs (see Table III), each gel consisted of 10 lanes and was loaded in the following manner: lanes 1 and 10, GP control; lane 2, GP plus wild-type HPr; lanes 3–9, GP plus the specified mutant HPrs. After electrophoresis, a xerographic copy of the stained gel (magnification: × 4) was used for measurement of gel shifts. A reference line was drawn to connect the bands in lanes 1 and 10 (glycogen phosphorylase controls). The distance (in mm) was measured between the gel-shifted bands and the reference line, and from these values, a ratio was calculated for the distance the mutant HPr shifted GP in comparison with the shift produced by wild-type HPr.

**TABLE I**

**Purification of E. coli glycogen phosphorylase**

The total activity, total amount of protein, and specific activity were determined for the soluble steps of the purification scheme for GP. See “Experimental Procedures” for details of the purification and the GP assay. Chen and Segel (41) described the purification of GP and calculated that the activity of a purified preparation of the enzyme would be 1.2 units/mg. Their assays were performed in the direction of glycogen synthesis, at 37 °C (expected to exhibit twice as much activity as the present assays carried out at 25 °C), in the presence of 5′-AMP (expected to increase the activity by a factor of 2), and in the presence of added Na2SO4 (expected to increase activity by a factor of 10). Correction for these modifications to the assay methodology should result in a specific activity of 0.03, approximately 5% of the activity shown in Table I. Yu et al. (13) described the purification of GP after hyperexpression from a plasmid. Their assays were performed in the direction of glycogen synthesis, at 30 °C, and in the presence of 5′-AMP. Correction for the differences in methodology of their reported specific activity (0.19 units/mg) should result in a specific activity of 0.06, approximately 10% of the activity shown in Table I. Assuming that GP is equally active when assayed in either direction, the present preparation is 10–20 times more active than previously reported.

| Purification step | Total activity | Total amount of protein | Specific activity |
|-------------------|----------------|-------------------------|------------------|
|                   | units          | mg                      | units/mg         |
| Crude extract     | 16.33          | 214.90                  | 0.076            |
| Mono-Q column     | 9.17           | 21.27                   | 0.431            |
| Superose 12 column| 9.5            | 16.24                   | 0.585            |

**FIG. 2.** Real time interaction analysis of proteins with immobilized HPr. Crude extract of E. coli IF-1 (100 μg protein/ml) (sensorgram a), purified protein factor (8 μg/ml) (sensorgram b), and crude extracts (10 μg protein/ml) of E. coli strain SB221/pJF02 before (sensorgram c) and 4 h after (sensorgram d) expression of glycogen phosphorylase by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (dissolved in the standard running buffer) were sequentially allowed to flow over the HPr surface to test for binding as described under “Experimental Procedures.” The arrows indicate the starting points of the injections.
ryl acceptance from EI and the sequential phosphoryl transfer to enzymes IIA, it seemed possible that the detected interaction might be due to formation of HPr-EI or HPr-IIAGlc complexes. When purified EI or IIAGlc (600 μl of 10 μg/ml of each protein) was exposed to the HPr surface for 10 min in the BIACore, no interaction was detectable (data not shown). Furthermore, in vitro phosphorylation of EI or IIAGlc by preincubation with P-enolpyruvate (for EI) and catalytic amounts of EI and HPr (for IIAGlc) did not promote binding to immobilized HPr (data not shown). It is worth noting that the association constant between EI and HPr was recently reported to be about 10$^5$ M$^{-1}$ by isothermal titration calorimetry (30). It is unlikely that an interaction with a $K_a$ in this range would be detected by SPR.

Therefore, the observed interaction with crude extract suggested that there is another endogenous *E. coli* protein that binds to HPr, presumably with a higher affinity than either enzyme I or IIAGlc.

**Purification of the Binding Factor**—The approach to purification of the factor that interacted with HPPr was based on SPR analysis; fractions from each purification step were examined for binding to immobilized HPr using the BIACore. *E. coli* strain IF-1 was grown in 2 liters of Luria broth at 37 °C to early stationary phase. The harvested cell pellet was washed and resuspended in buffer A (25 mM Tris·HCl buffer, pH 7.5, containing 100 mM NaCl); this suspension was passed twice through a French press at 10,000 p.s.i. The lysate was ultracentrifuged at 100,000 × g for 90 min to remove cell debris, and the supernatant solution was fractionated by ammonium sulfate precipitation. The fractions (30–40% saturation) showing binding affinity toward immobilized HPr were combined, dialyzed against buffer A, and chromatographed through a DE-52 column (25 × 300 mm) using a gradient of 100–500 mM NaCl (1 liter). Fractions eluting around 0.4 M NaCl demonstrated interaction with immobilized HPr; these fractions were pooled and concentrated in a 10 K Macrosep centrifugal concentrator (Filtron). The concentrated pool was further purified on a Superose 12 column (1.6 × 50 cm) using buffer A as an eluent. These fractions were analyzed, pooled, and concentrated as described for the DE-52 chromatography. At this step, the HPPr-binding protein was estimated by SDS-PAGE to be about 100 kDa in size and approximately 50% pure. As a final purification step, a phenyl-Superose 10/10 column (Pharmacia) was used with 10 column volumes of ammonium sulfate gradient (1–0 M) in 25 mM Tris-HCl buffer, pH 7.5. The fractions at the end of the gradient, which showed interaction with HPr, were pooled and concentrated. The purity was about 90% as judged by 4–20% SDS-PAGE (NOVEX).

This nearly homogeneous protein was tested for binding to different immobilized protein surfaces prepared as described under “Experimental Procedures.” The protein factor (8 μg/ml) was allowed to flow (rate of 10 μl/min) over each surface for 10 min. The protein showed the expected binding to immobilized HPr (Fig. 2, sensorgram b) but not to immobilized EI or IIAGlc (data not shown). A blank surface (see “Experimental Procedures”) also showed no interaction with the purified protein factor.

*The HPPr-binding Protein Is Glycogen Phosphorylase*—The purified binding protein was subjected to proteolysis (trypsin or endoproteinase Lys-C) following by sequencing analysis (see “Experimental Procedures”). The resultant sequences were processed by FASTA analysis to determine their identity to known sequences in the SWISS-PROT data base.

Selected fractions from the RP-HPLC separation of the trypsin digest were further purified by a second RP-HPLC step. Tryptic peptides, eluting at 72.2 and 74.6 min on the second column, were subjected to automated Edman degradation. The following amino acid sequences, respectively, were found: XY-

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VDCQDKVDELYELQEEWTAK and ESPDYXLEYGNPXEFK.
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*FIG. 3. Competition by soluble HPRs and other proteins for the interaction of glycogen phosphorylase with immobilized HPr.* Several concentrations of *E. coli* HPs were mixed with glycogen phosphorylase (2 μg/ml) in standard running buffer supplemented with 1 mM MgCl₂ and 1 mM dithiothreitol. One set of HPPr samples was phosphorylated by inclusion of 1 mM P-enolpyruvate and 1 μg/ml of EI in the incubation mixture. The samples were then allowed to flow over a surface containing immobilized HPr in the BIACore instrument. The increase in response units over a 10-min period relative to the control (absence of added HPPr) was plotted on a semilogarithmic scale. A competition test was also performed with a fixed concentration (2 μg/ml each) of the following proteins: *B. subtilis* HP (B-HP); *M. capricolum* HP (M-HP); *E. coli* enzyme I (EI); *E. coli* enzyme IIAGlc (IIAGlc).

The observed sequences represented fragments expected from cleavage on the carbonyl side of basic residues (Arg or Lys; R or K). FASTA analysis revealed the sequences to be identical to residues 762–783 and 167–182 of *E. coli* GP (p13031 in the SWISS-PROT data base).

The RP-HPLC separation of the endoproteinase Lys-C digest (see “Experimental Procedures”) yielded peaks that were mixtures of peptides. Upon amino acid sequencing, several of these peaks yielded multiple sequences that were later deduced to be identical to sequences in *E. coli* GP (data not shown). The correspondence of the sequences as well as the molecular weight of the HPPr-binding protein to *E. coli* GP suggested strongly that the HPPr-binding factor is GP.

*Overproduction of E. coli Glycogen Phosphorylase Increases Binding of Crude Extract to Immobilized HPPr*—It was anticipated that, if the HPPr-binding factor were GP, overexpression of that protein would result in enhanced HPPr binding to crude extracts. Strain SB221 harboring pJF02, an expression vector for GP, was used for this purpose. Fig. 2 shows sensorograms obtained by monitoring the flow of crude extracts (600 μl of 10 μg/ml of protein) from cells before induction (sensorogram c) and 4 h after induction with 1 mM IPTG (sensorogram d) (13). Crude extract from induced cells showed a much greater interaction with immobilized HPPr; this data provides further support for the idea that the HPPr-binding protein is glycogen phosphorylase.

*The Interaction between HPPr and Glycogen Phosphorylase Is Highly Specific*—Since *E. coli* GP shares a high degree of identity (greater than 40%) in amino acid sequence with GP from other organisms and *E. coli* maltodextrin phosphorylase (17), it was of interest to test the binding of HPPr to those proteins. Purified *E. coli* GP showed high affinity binding, but neither rabbit muscle phosphorylase a or b nor *E. coli* maltodextrin phosphorylase showed any affinity toward an HPPr surface (data not shown for *E. coli* maltodextrin phosphorylase; see Figs. 4 and 5 for rabbit enzymes).
A competition approach was taken to evaluate the specificity of the protein interaction with GP. Fig. 3 shows that if free E. coli HPr was included in the test solution with GP, the binding of GP to immobilized HPr was reduced. E. coli GP (2 μg/ml) was preincubated with the following proteins (2 μg/ml) in the standard running buffer: B. subtilis HPr (B-HPr) or M. capricolum HPr (M-HPr), E. coli EI (EI), E. coli IIA\textsuperscript{Glc} (IIA\textsuperscript{Glc}), or E. coli HPr (HPr), and each mixture was allowed to flow (10 μl/min) over the immobilized HPr surface. The response units (10-min monitoring period) were compared with the signal obtained when GP was tested alone. E. coli HPr competed with immobilized HPr for the binding with GP and gave about 60% competition compared with the control. Preincubation of HPr (10 μg/ml) with 2 μg/ml GP gave about 95% competition (Fig. 3). However, HPrs from other bacterial strains did not compete for GP binding. Neither EI nor IIA\textsuperscript{Glc} at either 2 μg/ml (Fig. 3) or 10 μg/ml (data not shown) influenced the binding of GP to immobilized HPr, as expected from the results of direct binding to immobilized HPrs. It should be noted that if GP bound to EI and/or IIA\textsuperscript{Glc} at unique sites in addition to binding to HPr, then the response would have been enhanced. These data support the conclusion that the binding of E. coli GP is highly specific for E. coli HPr.

Phosphorylation of HPr Enhances Its Interaction with Glycogen Phosphorylase—Frequently, the phosphorylation state of a regulatory protein modulates the activity of its interacting partner (2). Accordingly, the effect of phosphorylating HPr on its affinity for GP was checked by a competition experiment (Fig. 3). When HPr (0.2 μg/ml) was preincubated with GP under conditions where it became phosphorylated (see Fig. 3 legend), it gave about a 30% decrease in the signal for GP binding, while 2 μg/ml of phosphorylated HPr gave a 97% decrease in binding. The competition with phosphorylated HPr was significantly greater than with unphosphorylated HPr. A comparison of the two competition curves suggests that phosphorylated HPr (P-HPr) has about 4 times higher affinity toward GP than dephospho-HPr. The observation that P-HPr competes with HPr for binding to GP indicates that P-HPr binds to the same or an overlapping site on GP as does HPr. When EI was phosphorylated by incubation with P-enolpyruvate, data not shown), it had no effect on the binding of GP to immobilized HPr (data not shown), suggesting that immobilized HPr could not be phosphorylated.

Kinetic Analysis of the Glycogen Phosphorylase-HPr Interaction—Kinetic parameters for the binding of HPr to GP were determined using HPr immobilized to a sensor chip in the BIAcore system. Five different concentrations (1, 2, 4, 8, and 16 μg/ml) of purified GP were used for the binding analysis (Fig. 4). The signal increased as a function of analyte (GP) concentration. As a control, rabbit muscle GP (8 μg/ml) gave no response (see legend to Fig. 4). The dissociation constant (K\textsubscript{D}) for the HPr-GP interaction, using the BIAevaluation 2.1 software, was determined to be approximately 1.7 × 10\textsuperscript{-8} M, assuming interaction of the monomeric forms of both GP and HPr.

Interaction between HPr and Glycogen Phosphorylase Results in an Electrophoretic Mobility Shift—The biomolecular interaction studies, using the BIAcore, indicated a high affinity interaction of HPr and GP. It was therefore anticipated that such a stable complex might survive under electrophoretic conditions. Consequently, gel shift experiments using nondenaturing polyacrylamide gel electrophoresis (Fig. 5) were performed. While E. coli HPrs significantly retarded the mobility of GP in the gel (compare lane 1 or 10 with lane 2 or 9 in Fig. 5), HPr from M. capricolum (lane 7) or B. subtilis (lane 8) did not lead to a mobility shift of GP in the gel. To confirm the species specificity of phosphorylase binding to E. coli HPr, two different forms of rabbit muscle phosphorylase were run on the gel. Neither phosphorylase binding to a (lanes 3 and 4, respectively) nor b (lanes 5 and 6, respectively) showed an HPr-dependent gel shift. This is consistent with the finding that rabbit muscle GP shows no evidence for interaction with HPr by SPR (Fig. 4).

The Oligomeric State of Glycogen Phosphorylase Is Modulated by HPr—Sedimentation equilibrium experiments were carried out to evaluate the possibility that HPr binding to GP is associated with a structural change. The results of the various ultracentrifugal experiments are summarized in Table II. From the first pair of experiments, it is apparent that the binding of HPr to E. coli GP enhances monomer-dimer association almost 4-fold and dimer-tetramer association 66-fold. Mutant HPrs, defective in interaction with GP (Table III), show significantly different associative behavior with GP. These mutant forms of HPr do appear to be bound on the basis of a significantly better sum of squares for the fit of the data to the binding model when compared with the sum of squares for the fit to the nonbinding model. For the K40A mutant, the mono-
mer-dimer association is about 500-fold weaker, while the dimer-tetramer association is 2-fold stronger than for wild-type HPr. For the K72E mutant, the association for dimer formation is over 300-fold weaker, while that for tetramer formation is virtually the same as for wild-type HPr.

In contrast to *E. coli* GP, rabbit muscle GP exhibits a different mode of association, undergoing a monomer-tetramer-octamer association in both the absence and presence of HPr. It was not possible to determine whether or not HPr was bound on the basis of the fitting statistics. However, the effect of the presence of HPr on the associative behavior of the mammalian enzyme in Tris buffer is quite minimal; at best about a 2-fold

### Regulation of Glycogen Phosphorylase by HPr

#### Interaction of mutated HPrs with glycogen phosphorylase

Wild-type and mutant HPrs were examined for binding of HPr to glycogen phosphorylase (gel shift analysis) and activation of GP (GP activation assays). Gel shift results are recorded as the ratio of the distance that a mutant HPr shifted GP in comparison to the shift observed with wild-type HPr (see “Experimental Procedures”). A statistical analysis (using Origin, version 4.0, Microcal) showed a mean gel shift by wild-type HPr of 3.0 ± 0.6 (S.D.) (see Fig. 7). A statistical analysis (using Origin, version 4.0, Microcal) of the activation of GP by wild-type HPr showed a mean activation ratio (+HPr/–HPr) of 2.5 ± 0.26 (S.D.) (n = 18). The activation data for those mutations exhibiting a large decrease (≥50%) in GP activation are highlighted in boldface type.

### TABLE III

**Association properties of glycogen phosphorylase**

| Sample       | Buffer | Association model | $\Delta G^\circ$ | $K_D$ (mol) | Comment$^a$ |
|--------------|--------|-------------------|-----------------|-------------|-------------|
| GP           | Tris   | 1 ↔ 2 ↔ 4         | −11.40 (1 ↔ 2)  | 2.19 × 10^−10 (2 ↔ 1) |             |
| GP + HPr     | Tris   | 1 ↔ 2 ↔ 4         | −6.54 (2 ↔ 4)   | 1.34 × 10^−5 (4 ↔ 2) | HPr bound   |
| GP + HPr (K40A) | Tris   | 1 ↔ 2 ↔ 4         | −8.98 (2 ↔ 4)   | 8.49 × 10^−10 (2 ↔ 1) | HPr bound   |
| GP + HPr (K72E) | Tris   | 1 ↔ 2 ↔ 4         | −9.06 (2 ↔ 4)   | 1.99 × 10^−7 (4 ↔ 2) | HPr bound   |
| GP (rabit)   | Tris   | 1 ↔ 4 ↔ 8         | −8.92 (1 ↔ 2)   | 2.64 × 10^−7 (2 ↔ 1) | HPr bound   |
| GP (rabit) + HPr | Tris   | 1 ↔ 4 ↔ 8         | −9.06 (1 ↔ 2)   | 7.23 × 10^−6 (2 ↔ 1) | HPr not bound|

$^a$ Comment refers to the model fit.

$^b$ Tris buffer corresponds to 25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol.

### TABLE II

**Interaction of mutated HPrs with glycogen phosphorylase**

Wild-type and mutant HPrs were examined for binding of HPr to glycogen phosphorylase (gel shift analysis) and activation of GP (GP activation assays). Gel shift results are recorded as the ratio of the distance that a mutant HPr shifted GP in comparison to the shift observed with wild-type HPr (see “Experimental Procedures”). A statistical analysis (using Origin, version 4.0, Microcal) showed a mean gel shift by wild-type HPr of 3.0 ± 0.21 nm (S.D.) (n = 9). The gel shift data for those mutations exhibiting a ratio of mutant to wild-type of ≳0.3 are highlighted in boldface type. GP activation data represents the ratio of the stimulation of GP activity by a mutant HPr compared to the activation by wild-type HPr (see “Experimental Procedures”). Numbers in parentheses correspond to the activation ratio at a lower concentration of HPr (1 µg/ml) than that used in the standard assay (10 µg/ml) (see Fig. 7). A statistical analysis (using Origin, version 4.0, Microcal) of the activation of GP by wild-type HPr showed a mean activation ratio (+HPr/–HPr) of 2.5 ± 0.26 (S.D.) (n = 18). The activation data for those mutations exhibiting a large decrease (≥50%) in GP activation are highlighted in boldface type.

#### TABLE III

**Association properties of glycogen phosphorylase**

| Sample       | Buffer | Association model | $\Delta G^\circ$ | $K_D$ (mol) | Comment$^a$ |
|--------------|--------|-------------------|-----------------|-------------|-------------|
| GP           | Tris   | 1 ↔ 2 ↔ 4         | −11.40 (1 ↔ 2)  | 2.19 × 10^−10 (2 ↔ 1) |             |
| GP + HPr     | Tris   | 1 ↔ 2 ↔ 4         | −6.54 (2 ↔ 4)   | 1.34 × 10^−5 (4 ↔ 2) | HPr bound   |
| GP + HPr (K40A) | Tris   | 1 ↔ 2 ↔ 4         | −8.98 (2 ↔ 4)   | 8.49 × 10^−10 (2 ↔ 1) | HPr bound   |
| GP + HPr (K72E) | Tris   | 1 ↔ 2 ↔ 4         | −9.06 (2 ↔ 4)   | 1.99 × 10^−7 (4 ↔ 2) | HPr bound   |
| GP (rabit)   | Tris   | 1 ↔ 4 ↔ 8         | −8.92 (1 ↔ 2)   | 2.64 × 10^−7 (2 ↔ 1) | HPr bound   |
| GP (rabit) + HPr | Tris   | 1 ↔ 4 ↔ 8         | −9.06 (1 ↔ 2)   | 7.23 × 10^−6 (2 ↔ 1) | HPr not bound|

$^a$ Comment refers to the model fit.

$^b$ Tris buffer corresponds to 25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol.
HPr Specifically Activates E. coli GP—A phosphoglucomutase- and glucose-6-phosphate dehydrogenase-coupled enzyme assay (see "Experimental Procedures") was used to determine the effect of HPr on GP activity. This assay measures the activity in the physiological direction (glycogen degradation). The glucose 1-phosphate produced by GP promotes the activity in the physiological direction (glycogen degradation). The glucose 1-phosphate produced by GP promotes the conversion by the coupling enzymes of NADP to NADPH (detected by the change in absorbance at 340 nm).

The activity of E. coli GP was substantially increased by E. coli HPr (to 250% of the control value) (Fig. 7). The stimulation of GP activity elicited by E. coli HPr was not observed with HPs from B. subtilis or M. capricolum (data not shown). Other purified PTS proteins from E. coli (EI and IIA\textsuperscript{Glc}) also had no effect on the GP activity. Further, phosphorylation of EI by P-enolpyruvate (1 mM) did not affect GP activity (data not shown). None of the E. coli PTS proteins, HPr, IIA\textsuperscript{Glc}, EI, or P-EI had a significant effect on the activity of rabbit muscle GP a (active form) or b (inactive form) (data not shown). Therefore, E. coli HPr specifically activates E. coli GP.

HPr Modifies the Kinetic Behavior of GP—The stimulation of GP activity by HPr was saturable (Fig. 8A); 1 \mu g/ml of HPr gave 50% of maximal activation, while maximal activation was observed at 5 \mu g/ml.

The effect of HPr interaction with GP on the kinetic parameters was evaluated. When the concentration of glycogen was varied up to a concentration of 1% (Fig. 8B), there was no indication, as previously observed (13), that GP activity was approaching saturation. HPr (10 \mu g/ml) appeared to increase the affinity for glycogen. Consequently, the relative activation of GP by HPr was greater at lower glycogen concentrations. A reciprocal plot (Fig. 8B, inset) indicated that HPr decreased the $K_m$ for glycogen about 5-fold.

The concentration of phosphate was varied up to 80 mM (Fig. 8C). The response pattern was different from that observed for variation of glycogen concentration. In this case, HPr addition (10 \mu g/ml) resulted in a similar stimulation of the activity (to approximately 250% of the control) at all phosphate concentrations studied. This indicates that HPr affects the $V_{max}$ for phosphate. A reciprocal plot (Fig. 8C, inset) indicated that HPr increased $V_{max}$ (~5-fold).

Phosphorylation of HPr Abolishes Its Stimulatory Activity—P-enolpyruvate (1 mM), EI (2 \mu g/ml), and HPr were added to the coupled enzyme assay to determine if phosphorylation of HPr would affect its stimulatory activity. This reaction mixture was preincubated for 10 min before the assay was initiated by the addition of GP. The assay mixture containing P-HPr exhibited approximately 20% as much stimulation of GP activity as dephospho-HPr (Fig. 8A). The addition of IIA\textsuperscript{Glc} (1 \mu g/ml) to the incubation mixture containing P-HPr (resulting in the accumulation of P-IIA\textsuperscript{Glc}) did not change the degree of stimulation (data not shown). In the experiment shown in Fig. 8B, P-HPr addition resulted in no stimulation of GP activity at any concentration of glycogen tested, while the study in Fig. 8C showed P-HPr to have approximately 50% as much stimulation as dephospho-HPr at all concentrations of phosphate tested. Since it is difficult to reproducibly generate a sample of P-HPr totally devoid of the dephospho-form, it is likely that the variability in apparent activation by P-HPr is a reflection of the degree of contamination with the dephospho-form and that P-HPr itself does not activate GP.

Mutation of HPr Abolishes Its Regulatory Activity—To deter-
mine which amino acids in HPr are important for the binding and activation of GP, 51 mutant HPrs were examined by both gel shift and GP activity analysis. The gel shift results (Table III) are presented as the ratio of the distance that a mutant HPr shifted the GP band relative to the distance that wild-type HPr shifted the GP band. The GP activation data are expressed as the ratio of the increase in GP activity due to a mutant HPr relative to the activity increase observed with the wild-type HPr. GP activation by HPrs was tested at two levels: 1 \mu g/ml, a level that leads to 50% of maximal activation by wild-type HPr (data not shown); and 10 \mu g/ml, a level that results in maximal activation of GP (Fig. 8A and Table III).

Several of the mutant HPr proteins (R17G, R17E, K24Q, K24E, K27E, K27S, K40A, S46D, and Q51E), shown in boldface letters in Table III, produced gel shift ratios of \pm 0.3. In the case of Arg-17, mutation to Lys maintained the wild-type pattern (gel shift ratio = 0.9), indicating that a positive charge in that region is important for production of a complex with properties similar to that of wild-type HPr. The differences in the gel shift behavior of the three mutations at Arg-17 were paralleled by their capabilities to activate GP. The R17K protein elicited both a greater gel shift response and a greater activation of GP than did either the R17G or the R17E mutant HPr.

Mutation of HPr at Lys-24 to either Gln or Glu essentially abolished the capability of the HPr to produce a gel shift. Further, there was a drastic reduction in the capacity of these mutated HPrs to promote activation of GP. The K24E mutant was essentially inactive, and the K24Q mutant required a concentration of 10 \mu g/ml to produce approximately 60% as much activation response as wild-type HPr. Using a concentration of HPr of 1 \mu g/ml, the K24Q mutant HPr was only 10% as effective as wild-type HPr (see numbers in parentheses, Table III), suggesting that the affinity of this mutant protein for GP is considerably reduced. It appears from this analysis that Lys-24 is very important in the binding to and activation of GP.

Two mutations at Lys-27 (K27E and K27S) were examined; however, the S46R protein produced a complex that shifted in gels more than did the wild-type HPr-GP complex, and the S46E HPr-GP complex shifted only 60% as much as the control. The S46D HPr was unique in that it exhibited only a small gel shift (30% of the wild-type shift) and only a slight activation of GP. These assays indicate that Ser-46 plays an important role in the activation of GP and that, depending on the mutation, a variety of complexes with different properties in the gel shift assay can form.

Two mutant forms of HPr with changes in Gln-51 behaved differently in the two test systems. When the Gln residue was changed to Thr, the HPr behaved exactly as did the wild-type HPr. However, when the change was to Glu, the HPr-GP com-
The activity in $A$ is expressed as the increase over the basal activity of the complex shifted only 20% as much as did the complex with wild-type HPr. The effects of the mutation to Glu on GP activation were also substantial. Activation ratios of 10% at 1 $\mu$g/ml HPr and 50% at 10 $\mu$g/ml HPr were observed. This was a behavior similar to that of some of the other mutant proteins such as K40F, where the affinity for GP appears to be considerably decreased by the mutation. These determinations suggested that, while Gln (a polar residue) at position 51 of HPr is not essential for appropriate interaction with GP, substitution with negatively charged residues, such as Glu or Asp, leads to important changes in the nature of the interaction with GP.

The Lys residue at position 72 of HPr shows a unique behavior upon mutagenesis. The gel shift is only slightly decreased (10–20%) as a result of replacement by Arg or Glu. However, there is a substantial difference in the behavior of the two mutants in the GP activation test. The K72R mutant shows a decrease of 20% in GP activation, but the K72E mutant is essentially incapable of activating GP. Sedimentation equilibrium analysis of the K72E mutant protein (Table II) suggested that it does interact with GP but does not have as pronounced an effect on the association properties of GP as does the wild-type HPr. Consequently, it appears that the presence of a basic residue at position 72 is important for effective activation of GP.

Interestingly, the C-terminal Glu (residue 85) of HPr appears to play an inhibitory role in the activation of GP. Replacement of this residue by Lys results in a 50% increase in the HPr-dependent stimulation. The resultant complex also shifts in a gel 1.4 times as great a distance as does the wild-type complex.

**DISCUSSION**

While there was no history suggesting a role for HPr in metabolic regulation in enteric bacteria, we had a theoretical rationale to explore this possibility. HPr is both a phosphocarrier and a regulator in Gram-positive bacteria. Further, the cellular concentration of HPr in *E. coli* is significantly higher than necessary for its role as a phosphocarrier. The small size of HPr (less than 10 kDa) makes it an ideal potential regulatory factor. This perspective stimulated us to embark on ligand fishing experiments aimed at detecting a protein(s) exhibiting high affinity binding to HPr. This search turned up a protein with the expected tight binding; after purification to near homogeneity, sequencing experiments identified glycogen phosphorylase as the target of the HPr interaction.

The interaction between GP and HPr is highly specific; only *E. coli* HPr, not HPrs from Gram-positive bacteria or mycoplasma, interacts with (Fig. 3) and activates (Fig. 7) *E. coli* GP. A comparison of HPr primary sequences (2, 31, 32) shows that enteric HPrs are very similar to each other (those from *E. coli* and *Salmonella typhimurium* are identical); however, they differ considerably from those of Gram-positive bacteria and mycoplasma except around the site of phosphorylation (His-15).

![Figure 8](image.png)

**FIG. 8.** Effects of varying concentrations of HPr, glycogen, and phosphate on GP activity. The amount of HPr (0–15 $\mu$g/ml) ($A$), the amount of glycogen (0.1–1%) in the presence and absence of HPr or P-HPr (10 $\mu$g/ml) ($B$), and the amount of phosphate (2.5–80 mM) in the presence and absence of HPr or P-HPr (10 $\mu$g/ml) ($C$) was varied in the coupled enzyme assay mixture to determine their effects on GP activity. The activity in $A$ is expressed as the increase over the basal activity (0.00346 units), whereas the data in $B$ and $C$ are presented as activities in units.
Alcaligenes eutrophus (39), which has been proposed to play a role in the regulation of poly-β-hydroxybutyrate metabolism (40), the possibility was considered that NPr might function in an analogous manner in E. coli by regulating GP activity. This was ruled out. While E. coli HPr stimulates GP activity, neither NPr nor DTP has any effect on E. coli GP activity.

To learn the importance of individual amino acid residues in HPr involved in the binding and activation of GP, 51 mutant HPrs were examined. Those residues where mutation resulted in a significant change in the gel shift of the HPr-GP complex and resulted in a stimulation of GP activity of less than 50% compared with wild-type HPr included Arg-17, Lys-24, Lys-27, Lys-40, Ser-46, Gln-51, and Lys-72. The x-ray crystallographic structure (8) shows that these residues are on the surface of HPr and distributed over a large area (Fig. 9). Five of the seven residues have a positive charge, while the other two amino acids are polar, suggesting that electrostatic interactions are important for assembly of the normal, active HPr-GP complex.

It is noteworthy that Ser-46 is the site of a regulatory phosphorylation in Gram-positive bacteria; thus, Ser-46 is important in regulatory functions in both Gram-positive and Gram-negative bacteria. Clearly, x-ray crystallographic studies of the GP-HPr complex and mutagenic studies on GP will enhance our understanding of the details of the interaction of GP with HPr.

Several lines of evidence indicate that the E. coli HPr-dependent allosteric regulation of GP is specific for the E. coli enzyme. Although eucaryotic GPs exhibit a high degree of sequence identity to E. coli GP (17), rabbit muscle GP does not interact with E. coli HPr as evidenced by SPR (Fig. 4), gel shift analysis (Fig. 5), or sedimentation equilibrium studies (Fig. 6); additionally, rabbit muscle GP activity is not stimulated by HPr.

Both GP and maltodextrin phosphorylase are involved in the breakdown of glycogen in E. coli. Since there is extensive sequence conservation in these two proteins from E. coli (17), it was important to determine whether HPr interacted with maltodextrin phosphorylase as well as glycogen phosphorylase. The absence of HPr binding to maltodextrin phosphorylase emphasizes the high degree of specificity of this protein-protein interaction.

As a consequence of hyperexpression of glgP, we were able to develop a simple purification scheme for E. coli GP. The specific activity of the purified protein was greater than that previously reported (13, 41) (see Table I) but still considerably lower than that of rabbit muscle GP. We have found that this purified GP preparation is allosterically activated by the phosphocarrier protein HPr. The binding of HPr to GP increases the activity to approximately 250% of the basal level. Double reciprocal plots show that HPr-dependent activation leads to an increase (approximately 5-fold) in the affinity for glycogen. When the concentration of phosphate is varied, HPr promotes an approximately 5-fold increase in the V_max. Sedimentation equilibrium studies show that HPr changes the state of oligomerization of GP.

The cellular concentration of HPr in E. coli is in great excess over that of GP (41, 42). The phosphocarrier function of HPr in the PTS allows the protein to exist in both phospho- and dephospho-forms. Substantial activation of GP by HPr requires that it be in the dephospho-form (Fig. 8A). Other studies (Fig. 3) indicated that P-HPr has a higher affinity for GP than does dephospho-HPr. It is therefore reasonable to assume that, in vivo, E. coli GP is always bound to HPr and that physiological

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perturbations that shift the ratio of HPr to P-HPr should lead to changes in the activity of GP. Some mutations in HPr that involve the change of a positively charged side chain to one with a negative charge (R17E, K24E, K27E, K72E) result in a decrease in the capability of the HPr to activate GP (Table III). Perhaps the conversion of dephospho-HPr to phospho-HPr reduces the potential for GP activation by a similar mechanism, presumably not favoring the optimally active conformation. It is worth noting that the residues Lys-24, Lys-27, and Ser-46 of HPr appear to be essential both for activation of GP and for the previously studied (4) interaction of HPr with GTP. These three residues occupy a limited surface region on HPr (see Fig. 9).

As is the case in higher organisms, bacterial glycogen probably serves as a reservoir of energy and carbon. The mechanism that regulates the activity of E. coli GP differs from that controlling rabbit muscle and yeast GP. Rabbit muscle GP b (inactive form) is converted to an active form (GP a) by either binding of 5'-AMP or covalent phosphorylation at Ser-14 by phosphorylase kinase (43). The N-terminal region of yeast GP is 39 amino acid residues longer than the rabbit muscle enzyme and lacks a serine at residue 14 (17). Instead, the phosphorylation occurs in this extended region at Thr-30 by either a phosphorylase kinase or a CAMP-dependent protein kinase, neither of which recognize the rabbit muscle enzyme (44, 45). The N-terminal region of E. coli GP is 10 amino acid residues shorter than the rabbit muscle enzyme and, like yeast GP, lacks the Ser-14 phosphorylation site (17). Until now, it was believed that E. coli GP is subject to only minimal activity regulation; in contrast to the previously reported 30–40% increase in E. coli GP activity by 2 mM 5'-AMP (13, 41), 5'-AMP only produced an approximately 10% increase in GP activity in our preparation. The studies presented here establish that the high affinity interaction of E. coli GP with HPr is the major basis for activity regulation.

The genes encoding enzymes involved in glycogen metabolism (glycogen synthase and glycogen phosphorylase) are linked on a single operon that is regulated by the CAMP-CRP complex. Consistent with the concept that CAMP levels rise in stationary phase (46), it has been reported that glg operon expression is increased in stationary phase (47). The coincident preferential accumulation of glycogen in stationary phase (14) may reflect the preponderance of HPr in the phospho-form. When stationary phase cells enter a new round of growth, a number of metabolic changes resulting in the preferential degradation of glycogen might occur. The decrease in cellular CAMP level characteristic of the entry into logarithmic growth phase (46) would turn off the expression of the glg operon, and glycogen synthase activity might preferentially decay. The uptake of a PTS sugar should promote a shift in the state of HPr in the direction of dephospho-HPr, resulting in an activation of GP (Fig. 10). Consequently, effective recovery from the stationary phase of growth may be enhanced by the energy derived from the utilization of stored glycogen (48).

In summary, the novel finding that the activity of E. coli GP is regulated by the phosphocarrier protein, HPr, has been described. The regulation of GP activity by the state of phosphorylation of HPr is proposed to be the bacterial analogy to the covalent phosphorylation-dephosphorylation cascade characteristic of eucaryotic GPs.

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