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The *Escherichia coli* Adenylyl Cyclase Complex: Requirement of PTS Proteins for Stimulation by Nucleotides†

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ABSTRACT: GTP, as well as other nucleoside triphosphates, stimulates the activity of *Escherichia coli* adenylyl cyclase in permeable cells; the stimulatory effect is lost when the cells are disrupted by passage through a French pressure cell. These data suggested that the allosteric regulation by GTP of adenylyl cyclase activity requires an interaction of the enzyme with other protein factors. Strains deleted for genes encoding proteins of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) failed to show an activity stimulation by GTP. With a view to localizing the site of interaction of GTP with the adenylyl cyclase complex, a variety of studies using purified PTS proteins were performed using the photoaffinity labeling reagent, 8-azidoGTP. These studies showed that 8-azidoGTP bound specifically to HPr. A species specificity study showed that the photoaffinity reagent labeled *E. coli* HPr but not HPr proteins from *Mycoplasma capricolum* or *Bacillus subtilis*. A variety of site-directed mutations of *E. coli* HPr were evaluated for interaction with GTP by photoaffinity labeling as well as by nuclear magnetic resonance; the results of these studies indicate that the lysine residues at positions 24 and 27, serine-46, the threonine at position 36, and the aspartate at position 69 are important for the binding of GTP to HPr. Molecular modeling has been used to formulate a model for the binding of GTP to HPr involving electrostatic interaction of the phosphate groups of the nucleotide with the side chains of lysine residues 27 and 45 and serine-43, interaction of the sugar with serine-46, and interaction of the base with lysine-24. From these data, it is hypothesized that the binding of GTP to HPr is required for the GTP-dependent stimulation of the activity of the adenylyl cyclase complex.

The adenylyl cyclase of *Escherichia coli* is an enzyme subject to a complex mode of regulation (Peterkofsky et al., 1993). An intrinsic aspect of the regulation depends on the condition of phosphorylation of the proteins of the phosphoenolpyruvate:sugar phosphotransferase system (Peterkofsky, 1977). The previous paper in this series (Peterkofsky & Gollop, 1993) described some features of the stimulation of adenylyl cyclase activity in permeable cells by nucleoside triphosphates (ATP, CTP, UTP, and GTP) (Stein et al., 1985); the most potent stimulator is GTP, which is not a substrate for the enzyme. The focus of the current study is on the mechanism of the nucleotide stimulation. It is established here that PTS[1] proteins are required for the allosteric activation by nucleoside triphosphates and also that the PTS protein HPr from *E. coli* has the capacity to bind a photoaffinity derivative of GTP, 8-azidoGTP. A combination of the study of the labeling pattern of a variety of HPr mutants and molecular modeling studies made it possible to devise a model for the binding of GTP to HPr. On the basis of these results, it is hypothesized that HPr, a component of a complex of PTS proteins with adenylyl cyclase, plays an important role in the allosteric stimulation of adenylyl cyclase activity by nucleotides.

EXPERIMENTAL PROCEDURES

Materials

All of the nucleotides used in this study were obtained from Boehringer-Mannheim (Indianapolis, IN). [α-^{32}P]ATP (~30 Ci/mmol) and [2,8-^{3}H]cAMP (30−50 Ci/mmole) were from New England Nuclear. [α^{32}P]8-AzidoGTP (AzGTP)

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[1] Abbreviations: 8-azidoGTP or AzGTP, 8-azidoguanosine 5′-triphosphate; PTS, phosphoenolpyruvate:sugar phosphotransferase system.

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Table 1: Bacterial Strains and Plasmids Used in This Study

strain or plasmid	relevant properties and derivation	origin
457	Δpts strain N2679 (Δpts I,crr) transformed with pDIA100	Gottesman, 1976; Liberman et al., 1985
647	LBE 2041 recA transformed with pDIA100	Peterkofsky & Gollop, 1993
pDIA100	carries gene for adenylyl cyclase (cya)	Roy & Danchin, 1982

(7.0 Ci/mmol) was from ICN. 8-AzidoGTP was from Research Products, Mount Prospect, IL. Toluene is a Baker analyzed product. All other chemicals were of analytical grade. Precast SDS−PAGE gels (4−20%) were from Novex.

E. coli. Strains used in this study are listed in Table 1. All strains used for the measurement of adenylyl cyclase were transformed with the plasmid pDIA100 (Roy et al., 1983), which expresses the gene for adenylyl cyclase. As a result, the level of adenylyl cyclase is approximately 10-fold higher in these strains than in the parent strains. The higher level of adenylyl cyclase activity permits more accurate determination of the enzyme activity.

PTS Proteins. Purified preparations of enzyme I, HPr, and enzyme IIA^{glc} were made from extracts of *E. coli* that contain plasmids which overproduce these proteins and purified as previously described (Reddy et al., 1991). HPr from *Bacillus subtilis* was a gift from Jonathan Reizer, UCSD. HPr from *Mycoplasma capricolum* was a gift from Peng-Peng Zhu of this laboratory. Site-directed mutants of HPr at residue serine-46 (S46A, S46D, S46E, S46N, and S46R)[2] were created by a polymerase chain reaction method (Landt et al., 1990). The mutagenic primers were prepared by standard phosphoramidite chemistry (Mateuci & Caruthers, 1981). The sequence of the reverse mutagenic primers was as follows (mutated codon is in bold and underlined): S46A, 5′-TAAACAG**CGC**TTTCGCGCTGG-3′; S46D, 5′-TAAACAG**ATC**TTTCGCGCTGG-3′; S46E, 5′-TAAACAG**CTC**TTTCGCGCT-3′; S46N, 5′-TAAACAG**GTT**TTTCGCGCT-3′; S46R, 5′-TAAACAG**TCT**TTTCGCGCT-3′. Creation and purification of all other mutants of HPr have been previously described (Sharma et al., 1991, 1993; Sharma, 1992; Anderson et al., 1991, 1993). Since HPr proteins from *M. capricolum* (Zhu et al., 1993) and *E. coli* (Saffen et al., 1987; De Reuse et al., 1985) are deficient in tryptophan and tyrosine, the concentration of these proteins was assessed by the procedure of Waddell (1956), which does not depend on the ultraviolet absorption of aromatic amino acids.

[2] HPr proteins mutated at residue 46 are being characterized and will be described separately. The mutation S46D in *E. coli* HPr mimics the S46D mutation in *B. subtilis* HPr (Reizer et al., 1989) in that it is a very poor substrate for enzyme I; thus, these mutations behave similarly to P-SerHPr, formed by an ATP-dependent phosphorylation of Ser46 and found in HPrs from Gram-positive species (Reizer et al., 1984, 1993). S46E is also impaired in respect to enzyme I. Because S46D and S46E are such poor enzyme I substrates, sugar-specific enzyme II reactions could not be assessed. S46R, S46N, and S46A give either wild-type kinetics or differences that do not approach the magnitude (several thousandfold inhibition) of that found for S46D (J. W. Anderson and E. B. Waygood, unpublished results). Initial structural investigations by both NMR (R. E. Klevit, unpublished results) and X-ray crystallography (S. Napper and L. T. J. Delbaere, unpublished results) show that there are only small changes in the structure of S46D, the significance of which is currently being investigated.

Methods

Growth and Permeabilization Conditions. Bacteria to be used for measurement of adenylyl cyclase activity were grown in salts medium (Vogel & Bonner, 1956) supplemented with 0.8% Difco nutrient broth and ampicillin (50 μg/mL) to maintain the plasmids. Where indicated, the medium was supplemented with cAMP (5 mM). Cultures were prepared for adenylyl cyclase assays as previously described (Harwood & Peterkofsky, 1975).

Adenylyl Cyclase Assay. Assays for adenylyl cyclase were performed immediately after permeabilization as described previously (Harwood & Peterkofsky, 1975). The assay mixture contained bicine buffer, pH 8.5 (20 mM), $MgCl_2$ (10 mM), dithiothreitol (1 mM), and radioactive substrate, [α-^{32}P]ATP, at a concentration of 0.1 mM or as indicated in the figure legends. Protein content was estimated by the method of Lowry et al. (1951). As shown in Figure 1, all assays were carried out at four time points. Rates were calculated by drawing the best line through the four time points.

PTS Assay. Enzyme I was assayed by the lactate dehydrogenase-coupled assay. HPr proteins mutated at residue 46 were assayed similarly and by a PTS sugar phosphorylation assay in which enzyme I was limiting, as previously described (Waygood et al., 1979).

Photoaffinity Labeling with 8-AzidoGTP. Photoaffinity labeling was carried out under conditions recommended by Potter and Haley (1983) and by personal communications from Boyd Haley. Reactions were carried out in a volume of 10 μL in 500 μL microcentrifuge tubes and contained 1.43 μM [α-^{32}P]AzGTP (0.1 μCi), 1 mM $MgCl_2$, 20 mM HEPES (pH 8.0), and 10 mM NaCl. Protein substrates for photolabeling were added at a concentration of 1 μg. For determination of the specificity of photoaffinity labeling (Figure 4), various amounts, as specified, of AzGTP or other nucleotides were added to the incubation mixtures. After incubation for 10 min at 4 °C with shaking to allow proteins to equilibrate with the AzGTP, the samples were photolyzed for 1 min [using a hand-held 4-W 254-nm lamp (UVG-11, UV Products, Upland, CA) at a distance of 3.1 cm]. The photolabeling time was standardized using HPr; it was found that the amount of photolabeling plateaued from 45 s to 2 min. Suitable controls indicated that there was no labeling of HPr in the absence of ultraviolet illumination. The light intensity was measured as 3400 μW/cm^2 at a distance of 3.1 cm using a model J-225 shortwave ultraviolet measuring meter (UV Products, Upland, CA). The reactions were stopped by the addition of equal volumes of SDS gel loading buffer [0.125 M Tris·HCl, pH 6.8, 20% glycerol, 4% (w/v) SDS, 0.005% bromophenol blue, 10 mM dithiothreitol, and 5 mM EDTA] which quenched the reaction. The samples were loaded on precast 4−20% Tris−glycine gradient gels (Novex) without the usual incubation in a boiling H_2O bath. In control experiments, it was found that boiling samples did not affect the amount of photolabeled HPr. Electrophoresis was run at 125 V for about 1.5 h at which point the dye front was approximately at the bottom of the gel. After electrophoresis, the gels were stained with Coomassie blue R-250, destained, dried, and exposed to Kodak XA125 film at −70 °C for autoradiography. The data of Figure 4A were generated by calculating radioactivity in the regions corresponding to HPr on dried gels using a Betascope 603 imaging

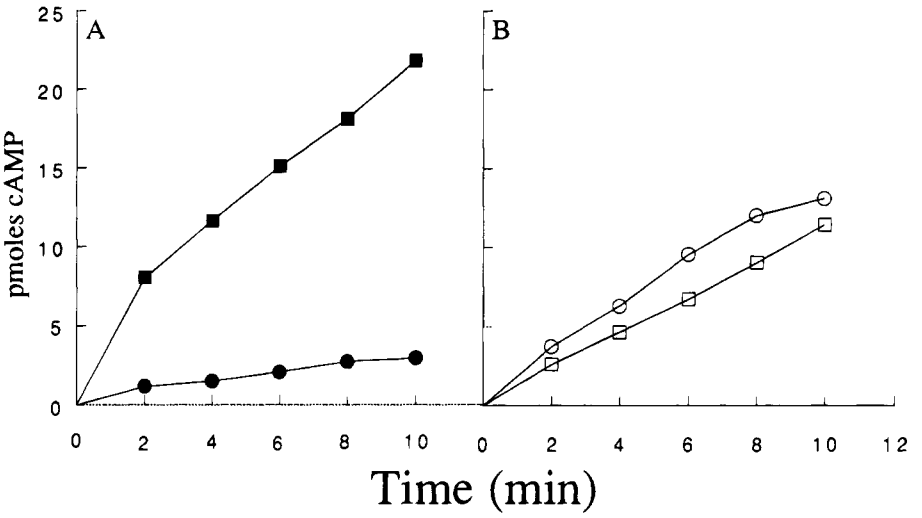

FIGURE 1: Effect of GTP on adenylyl cyclase activity in toluene-treated cells and cell extracts of pts$^+$ *E. coli*. A culture of strain 647 (pts$^+$, see Table 1), grown as described in Methods, was washed and resuspended in 100 mM potassium phosphate buffer, pH 8.5. The culture was divided into two parts, one of which was used for preparation of toluene-treated cells and the other for preparation of a cell extract by passage through a French pressure cell. Reaction mixtures (1.5 mL) containing 0.1 mM ATP as substrate for measurement of adenylyl cyclase activity (see Methods) were set up. At the indicated times, aliquots (0.1 mL) were removed for the determination of the amount of [^{32}P]cAMP formed. Each 0.1 mL aliquot of reaction mixture contained 0.044 mg of protein (toluene-treated cells) or 0.039 mg of protein (French press extract). Panels: (A) toluene-treated cells; (B) French press extract. Symbols: circles, GTP absent; squares, GTP (1 mM) present. Panel A is identical to Figure 1 of Peterkofsky and Gollop (1993) and is included for reference purposes. [Reprinted with permission from Peterkofsky and Gollop (1993). Copyright 1993 Cambridge University Press.]

system (Intelligenetics, Waltham, MA). The data in Figure 4B and Table 3 were accumulated by quantitation of the densities in the HPr regions of autoradiograms using the NIH Image program (version 1.55) developed at NIH by Wayne Rasband.

NMR Spectroscopy. All NMR experiments were performed on 1 mM protein samples in D_2O containing 50 mM potassium phosphate buffer and 0.2 mM EDTA. The pD of wild-type, S46R, and S46D HPrs was adjusted to 6.5 and monitored during the GTP titration. A 50 mg/mL stock solution of GTP in D_2O was made and kept on ice. Aliquots of this stock were added to the NMR samples, and a 1D spectrum was acquired for each successive addition of 0.25 equiv of GTP. All spectra were collected at 30 °C with 32 transients and a spectral width of 10 000 Hz. The HDO signal was suppressed by selective presaturation. The 1D GTP titration was continued up to 2 equiv of GTP per equivalent of protein, and the same sample was then used to collect 2D NOESY and TOCSY data. The mixing times of the NOESY and TOCSY experiments were 200 and 70 ms, respectively, and between 700 and 800 t_1 increments were acquired for each 2D experiment. The wild-type and S46D data were collected on a 500 MHz Bruker AM console whereas the S46R data were collected on a 500 MHz Bruker DMX console.

Spectra were processed using the software program Felix 2.0 (Biosym). For the 1D titration, each FID was multiplied by a sine-squared window function phase shifted by 65° followed by Fourier transformation. Baseline correction was done using a third-order polynomial. 2D spectra were zero-filled in both dimensions and multiplied by sine-squared window functions phase shifted by 65° in both t_1 and t_2. Using Origin (version 3.0, Microcal, Inc., Northampton, MA), the data from the chemical shifts of F48$^\delta$H and the F48$^\epsilon$H were fit to a third-order polynomial regression (see Figure 8).

Molecular Modeling. Modeling of the HPr·GTP complex was done with Quanta (Molecular Simulations, Waltham, MA) using the HPr structure 1poh.pdb (Jia et al., 1993) from the Brookhaven data bank. As a control, modeling was first done on HPr without GTP. All hydrogens were added to the structure, followed by solvation by an 8 Å shell of water. Using the CHARMm (Brooks et al., 1983) module in Quanta, the structure was then minimized (to <0.1 kcal/Å) and warmed from 0 to 500 K during 0.5 ps of dynamics. Dynamics continued at 500 K for an additional 4.5 ps, and then the structure was reminimized (to <0.01 kcal/Å).

The GTP molecule was bound "manually" to unsolvated HPr using the solid docking module of Quanta. The binding site was selected using experimental data on site-directed mutants from Table 3 and structural homologies to other nucleotide binding proteins. GTP was placed near K24, K27, and S46 and with the guanine base near F48 to provide perturbation to the phenylalanine ^{1}H resonance frequencies observed by NMR (Figure 8). After docking, the complex was resolvated, and the same dynamics/minimization protocol outlined above was performed. The S46R mutant of HPr was created with the molecular editor module of Quanta using the unsolvated HPr·GTP model structure after dynamics. The structure was then resolvated, and the dynamics and minimization were performed as outlined above.

RESULTS

Stimulation of Adenylyl Cyclase Activity by GTP. It was shown in our previous study (Peterkofsky & Gollop, 1993) that the adenylyl cyclase activity in *E. coli* could be stimulated by GTP and other nucleoside triphosphates. In the present work, an investigation was made of the requirements with respect to protein−protein interactions for the nucleotide stimulatory effect. It was observed (see Figure 1) that, while GTP promoted a substantial (roughly 10-fold) stimulation of the enzymatic activity in preparations of permeable cells from a wild-type strain of *E. coli* (panel

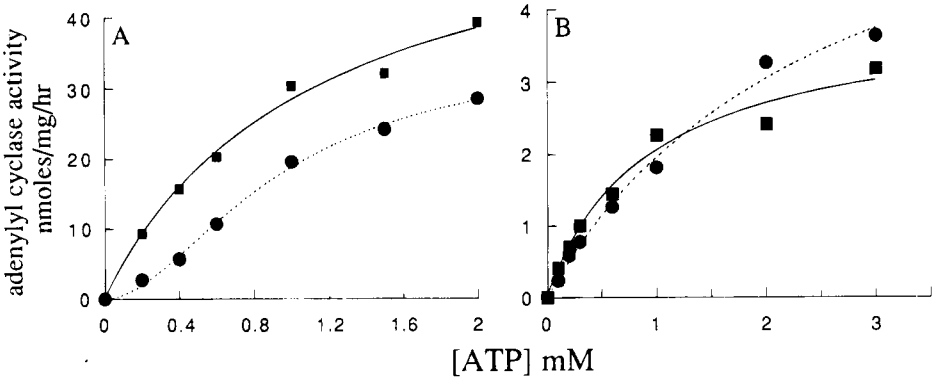

FIGURE 2: ATP concentration dependence for adenylyl cyclase activity in wild-type and PTS deletion strains of *Escherichia coli*. Toluene-treated cells of strains 647 (pts^+, panel A, see Table 1) and 457 (Δpts, panel B, see Table 1) were prepared as described under Methods. These preparations were tested for adenylyl cyclase activity (see Methods) at the indicated concentrations of ATP. Symbols: filled circles, GTP absent; filled squares, GTP (1 mM) present. The values of V, K, and n were calculated using the Enzfitter (version 1.03) program. These values were fit to the Michaelis–Menten equation using the Kaleidagraph (version 2.1; Synergy Software, Reading, PA) program to produce the curves shown. Each 0.1 mL aliquot of reaction mixture contained 0.028 mg of protein for strain 647 and 0.033 mg of protein for strain 457. Panel A is identical to Figure 3 of Peterkofsky and Gollop (1993) and is included for reference purposes. [Reprinted with permission from Peterkofsky and Gollop (1993). Copyright 1993 Cambridge University Press.]

A), the stimulatory effect was abolished in cell extracts (panel B). In fact, the data in Figure 1B suggest that, in cell extracts, GTP addition results in a small inhibition.

The loss of the capability of adenylyl cyclase to be stimulated by GTP in cell extracts indicated that the enzyme might require an interaction with some other macromolecule(s) to respond to GTP. Since there is evidence (Peterkofsky et al., 1993; Reddy et al., 1985; Peterkofsky, 1977) that adenylyl cyclase is influenced by interaction with one or more component(s) of the phosphoenolpyruvate:sugar phosphotransferase system (PTS), a comparison was made of the effect of GTP on adenylyl cyclase activity in permeable cell preparations of a pts^+ and a Δpts strain (Figure 2). As shown in panel A, a pts^+ strain (647, see Table 1) exhibited sigmoid velocity vs substrate plots in the absence of GTP and a hyperbolic plot in the presence of GTP. At low concentrations of substrate, GTP stimulates the activity approximately 5-fold. In contrast, the Δpts strain (457, see Table 1), deleted for genes for enzyme I and enzyme IIA^{glc}, showed hyperbolic kinetics in the absence or presence of GTP with no sign of a stimulatory effect of the nucleotide at any substrate concentration (panel B). These data suggest that an interaction of adenylyl cyclase with one or more of the PTS proteins is essential for the enzyme to exhibit the allosteric regulatory response to GTP.

Strains of *E. coli* carrying mutations in genes encoding enzyme I, HPr, or enzyme IIA^{glc} are known to have lower levels of cAMP than wild-type strains (Peterkofsky et al., 1978). It therefore seemed possible that the reason for the inability of the adenylyl cyclase in such strains to be stimulated by GTP (Figure 2, panel B) was that some non-PTS gene(s), required for the GTP stimulation effect, required cAMP for their expression. The data in Table 2 show that this model is not supported. The Δpts strain shows little, if any, stimulation of adenylyl cyclase activity in permeable cell preparations prepared from bacteria grown in the absence or presence of cAMP. In contrast, the pts^+ strain shows a stimulation of adenylyl cyclase activity by GTP in permeabilized cell preparations derived from bacteria grown in the absence (611% of control activity) or presence (1650% of control activity) of cAMP. It is likely that the enhancement of the GTP-dependent stimulation of enzyme activity by growth in medium supplemented with cAMP is due to the increased expression of some gene(s) activated by cAMP-CRP, perhaps the pts operon (Fox et al., 1992).

Table 2: Effect of GTP on Adenylyl Cyclase Activity in Wild-Type or PTS Deletion Strains Grown in the Absence or Presence of cAMP[a]

strain	GTP in assay	cAMP in growth medium	adenylyl cyclase activity (% of control)
pts^+	−	−	100
pts^+	+	−	611
pts^+	−	+	120
pts^+	+	+	1650
Δpts	−	−	100
Δpts	+	−	120
Δpts	−	+	77
Δpts	+	+	144

[a] Strains 647 (pts^+, see Table 1) and 457 (Δpts, see Table 1) were grown for measurement of adenylyl cyclase activity in permeable cells as described in Methods. Where indicated, the culture medium was supplemented with cAMP (5 mM). Activity was measured in the absence (control activity) or the presence of 1 mM GTP, using a concentration of radioactive ATP of 0.1 mM as substrate. The data are expressed as the percent of control activity observed when the assay was carried out in the absence of GTP for cells grown in the absence of cAMP. The control adenylyl cyclase activities expressed as picomoles of cAMP formed per milligram of protein per hour measured were 7285 (strain 647, pts^+) and 1468 (strain 457, Δpts).

PTS Proteins Interact with GTP. The data presented in Figure 2 suggested that stimulation of adenylyl cyclase activity by GTP required the presence of PTS proteins. No effect of GTP (concentrations to 5 mM) on the enzyme I–HPr reaction was found when either HPr or phosphoenolpyruvate concentrations were varied (data not shown). An independent approach was thus taken to evaluate the possible interaction of these proteins with GTP. The strategy taken was to measure the capability of purified enzyme I, HPr, and enzyme IIA^{glc} to become photoaffinity labeled with a photolabile derivative of GTP, 8-azidoGTP (AzGTP) (Figure 3). The results of this study indicated that HPr was labeled to a significantly greater degree than was either enzyme I or enzyme IIA^{glc}. When HPr was mixed with equivalent amounts of enzymes I and IIA^{glc} (lane 5), the amount of label incorporated into HPr was reduced to 40% of that seen in the absence of these proteins (lane 2). This

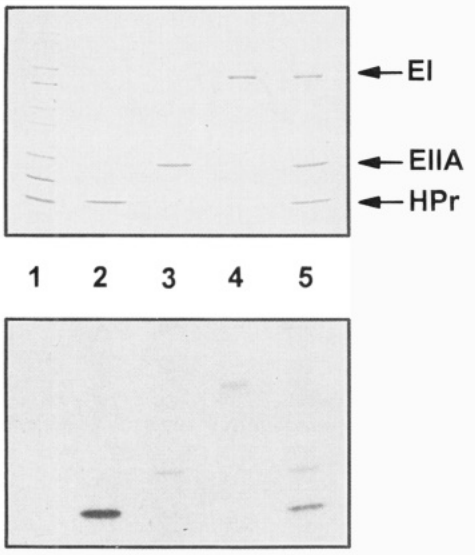

FIGURE 3: Photoaffinity labeling of purified PTS proteins. Purified preparations of the PTS proteins enzyme I (EI), enzyme IIA^{glc} (EIIA), and HPr were photoaffinity labeled with [α-^{32}P]AzGTP as described under Methods. The samples were electrophoresed on a 4−20% PAGE gel and stained with Coomassie blue (upper panel). The stained gel was then subjected to autoradiography (lower panel). Lanes: 1, molecular weight markers; 2, HPr; 3, enzyme IIA^{glc}; 4, enzyme I; 5, mixture of all three proteins. Approximately 1 μg of each protein was used.

reduction is probably a nonspecific protein effect (see Figure 5); the persistence of significant labeling of HPr in the presence of these proteins eliminates the possibility that the preparations of enzymes I and IIA^{glc} contain some inhibitor of photolabeling. The trace of labeling of enzymes I and IIA^{glc} is assumed to be due to nonspecific photolabeling.

The data presented in Figure 4A indicate that the photoaffinity labeling of HPr is saturable. Maximal binding of AzGTP requires approximately 1.5 mM AzGTP. Under these conditions, approximately 10% of the HPr is derivatized.

The nucleotide specificity for the photolabeling of HPr was examined (Figure 4B). The data indicate that the guanosine nucleotides GTP, GDP, and GMP were effective competitors and that the order of effectiveness was GTP > GDP > GMP. ATP, CTP, and UTP were approximately 50% as effective as GTP in competing for the photolabeling. The photolabeling was specific, since guanosine was essentially ineffective as a competitor.

Labeling of proteins with AzGTP is not very quantitative (Grammer et al., 1993). Incorporations from 0.01% to 50% have been observed with purified proteins (Potter & Haley, 1983). In order to eliminate the possibility that the AzGTP was reacting with an impurity in the HPr preparation, it was therefore felt important to further evaluate the purity of the HPr preparation used for photolabeling studies. Examination of the purity of the HPr preparation on SDS−PAGE with silver staining revealed only a single stained band (data not shown).

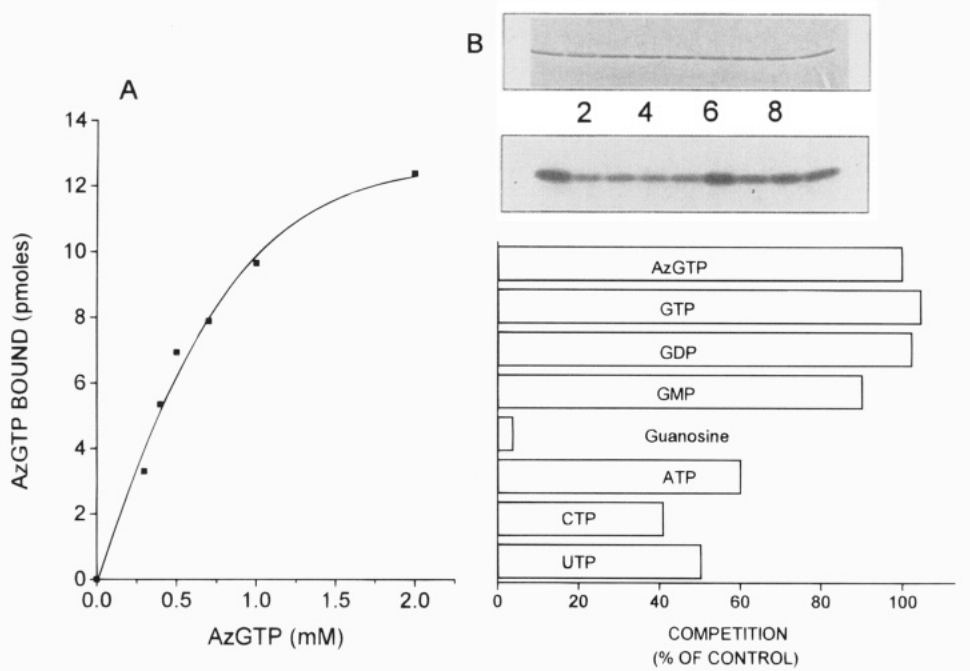

FIGURE 4: Specificity for photoaffinity labeling of HPr. (A) Dose−response curve for AzGTP. Samples of purified HPr (1 μg) were incubated for photoaffinity labeling with [α-^{32}P]AzGTP (final concentration = 1.43 μM) as described under Methods. The indicated concentrations of unlabeled AzGTP were included in the incubation mixtures. After SDS−PAGE, the gel was stained with Coomassie blue and dried. The radioactivity in the HPr region in each lane was quantitated by image analysis using a Betascope as described under Methods. From the radioactivity incorporated into HPr and the specific activity of AzGTP in the various incubation mixtures, the amount of AzGTP bound to HPr was calculated. The amount of HPr in the incubation mixtures corresponded to approximately 110 pmol of protein. (B) Competition by nucleotides for photolabeling of HPr. Samples of HPr (1 μg) were incubated for photoaffinity labeling with [^{32}P]AzGTP (final concentration = 0.4 mM). For testing competition, incubation mixtures were supplemented with 1.6 mM indicated nucleotide. After SDS−PAGE, the gel was stained with Coomassie blue, dried (upper panel), and subjected to autoradiography (center panel). The densities in the HPr regions of the autoradiogram were quantitated using NIH Image. The sample order was lane 1, no additions; lane 2, AzGTP; lane 3, GTP; lane 4, GDP; lane 5, GMP; lane 6, guanosine; lane 7, ATP; lane 8, CTP; lane 9, UTP. The control competition with 1.6 mM added unlabeled AzGTP reduced the incorporation to 56% of that observed at 0.4 mM AzGTP. This was taken as the control competition (100%). The bar graph shows the calculated competition for the indicated nucleotides.

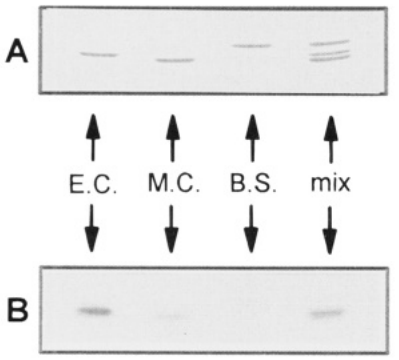

FIGURE 5: Species specificity of photoaffinity labeling of HPr. Purified HPr preparations (1 μg) from various sources were photoaffinity labeled with [α-^{32}P]AzGTP as described under Methods. Panels: (A) gel stained with Coomassie blue; (B) autoradiogram of the stained gel. Abbreviations: E.C., *E. coli* HPr; M.C., *M. capricolum* HPr; B.S., *B. subtilis* HPr; mix, mixture of the three HPr preparations.

The preparation of HPr used in these studies was purified from cells overexpressing the protein (see Experimental Procedures). We also examined the activity of a preparation of HPr purified from wild-type *E. coli* not hyperexpressing the protein (Beneski et al., 1982) and found this to be equally active in photoaffinity labeling with AzGTP (data not shown).

The previous studies indicated that, of the three soluble PTS proteins of *E. coli*, HPr was the only protein that interacted effectively with AzGTP. In order to see whether the reactivity of HPr with AzGTP was observable with all HPr proteins, a comparison of the capability to become photolabeled with AzGTP of HPr proteins from different species was carried out (Figure 5). Identical amounts (see stained gel, panel A) of purified preparations of HPr from *M. capricolum* (M.C.) and *Bacillus subtilis* (B.S.) showed only trace levels of labeling (panel B) compared to the *E. coli* (E.C.) HPr. When all three preparations of HPr were mixed (lane labeled mix), there was a 30% diminution of the label in *E. coli* HPr; this control eliminated the possibility that the *M. capricolum* and/or *B. subtilis* HPr preparations contained some component that inhibited the photolabeling reaction. The finding that only the HPr from *E. coli* reacts with AzGTP is a further indication of the specificity of the observed photolabeling reaction.

A large collection of site-directed mutants of *E. coli* HPr were evaluated for their capability to be photolabeled with AzGTP. The results summarized in Table 3 indicate that only a limited number of positions in the protein are essential for effective photolabeling. These are the lysine residues at positions 24 and 27, the threonine residue at position 36, serine-46, and aspartate-69 (these positions are highlighted in bold lettering in Table 3).

Figure 6 shows the photolabeling data for those mutants in the region of lysine-27. While the Q21E (lane 2), Q21T (lane 3), and S31A (lane 8) mutants are photolabeled comparably to the wild-type protein, the K24Q (lane 4), K24E (lane 5), K27E (lane 6), and K27S (lane 7) mutants are incapable of being photolabeled. The figure also shows the inactivity for labeling with the T36N (lane 9) and D69E (lane 10) mutants.

The data shown in Figure 7 deal with an analysis of a variety of site-directed mutants at serine-46. Change of the serine residue to alanine (lane labeled A) or asparagine (lane labeled N) results in retention of photolabeling capability; change to either aspartate (lane labeled D) or glutamate (lane labeled E) results in loss of the activity. It is interesting to note that, when the serine is changed to arginine (lane labeled R), the photolabeling is enhanced to a level approximately three times that of the control (see Table 3). It is worth noting that the solution (van Nuland et al., 1994) and crystal (Jia et al., 1993) structures of *E. coli* HPr indicate that positions K24, K27, and S46 are relatively close to each other (see Discussion).

Table 3: Photoaffinity Labeling of Site-Directed Mutants of *E. coli* HPr[a]

mutation	photoaffinity labeling (% of wild type)	mutation	photoaffinity labeling (% of wild type)
wild type	100	**S46E**	**22.3**
F2Y	81.5	**S46R**	**290.6**
Q3K	83	S46N	53.1
Q4S	112.3	S46A	84.9
E5Q	139.6	Q51T	75.7
T7N	82.8	Q51E	62.2
N12D	131.1	Q57E	62.8
R17G	79	T62N	65.2
R17E	63.8	T62A	72.7
R17K	90.9	S64Y	68.1
Q21E	106.2	E66K	104.9
Q21T	78.8	**D69E**	**16.2**
K24Q	**26.6**	E70K	132.9
K24E	**28.5**	Q71E	94.6
K27E	**19.4**	K72R	68.7
K27S	**32.6**	V74I	75.3
S31A	64.2	E75R	107.6
T34Q	105.3	H76A	60.4
T36N	**35.6**	L77I	81.1
N38D	69.6	K79E	63.4
K40A	65.2	A82E	75
K40F	56.2	A82S	88.9
S41K	124.7	E83A	128.7
S43N	85.9	Δ84	104.6
S46D	**22.3**	E85K	131.4

[a] Mutations in HPr were constructed and proteins purified as described in Methods. Purified preparations of the various HPr species (approximately 1 μg) were photoaffinity labeled with [α-^{32}P]AzGTP (1.43 μM) as described under Methods. The HPr samples were then electrophoresed as in Figure 5. The gels were then stained and subjected to autoradiography as described in Figure 5. The densities in the HPr regions of the autoradiograms were quantitated using NIH Image. Δ84 corresponds to deletion of residues 84 and 85. Mutated species in bold letters correspond to those proteins that react with [α-^{32}P]AzGTP significantly differently than does wild-type HPr.

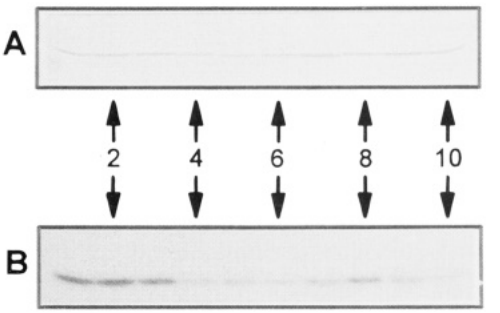

FIGURE 6: Photoaffinity labeling of site-directed mutants of *E. coli* HPr in the region of lysine-27. Purified preparations of mutated forms of HPr (approximately 1 μg) were photoaffinity labeled with [α-^{32}P]AzGTP as described under Methods. Panels: upper, gel stained with Coomassie blue; lower, autoradiogram of the stained gel. Lanes: 1, wild-type HPr; 2, Q21E mutant; 3, Q21T mutant; 4, K24Q mutant; 5, K24E mutant; 6, K27E mutant; 7, K27S mutant; 8, S31A mutant; 9, T36N mutant; 10, D69E mutant.

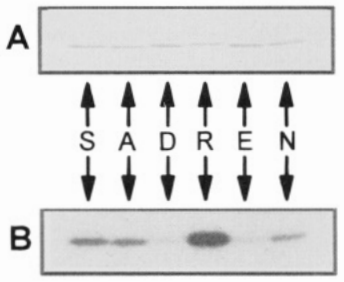

FIGURE 7: Photoaffinity labeling of site-directed mutants of *E. coli* HPr at serine-46. Purified preparations of mutated forms of HPr (approximately 1 μg) were photoaffinity labeled with [α-^{32}P]AzGTP as described under Methods. Panels: upper, gel stained with Coomassie blue; lower, autoradiogram of the stained gel. Abbreviations: S, wild-type HPr; A, S46A mutant; D, S46D mutant; R, S46R mutant; E, S46E mutant; N, S46N mutant.

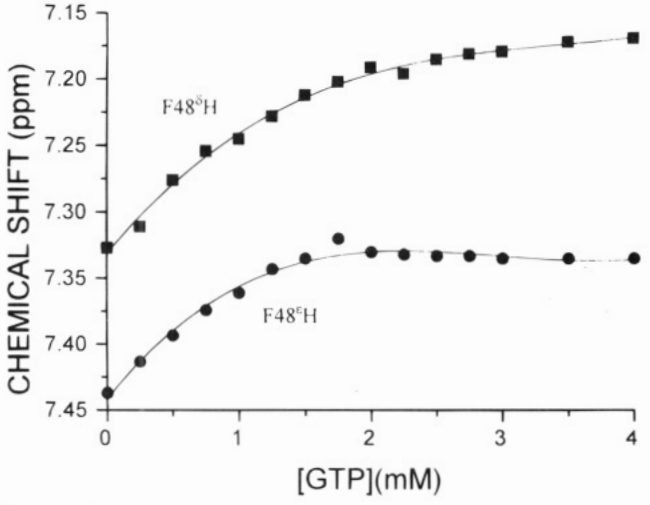

FIGURE 8: Titration of S46R HPr with GTP. NMR spectroscopy of the HPr in the absence and presence of increasing amounts of GTP was carried out, and the analysis of the data was performed as described under Methods.

Nuclear magnetic resonance spectroscopy has been used to characterize the solution structure of *E. coli* HPr. Experiments were carried out to measure the interaction of GTP with HPr by a nucleotide-dependent perturbation of the NMR spectrum of the protein (Figure 8). Titration of GTP into solutions of wild-type or S46R HPr resulted in shifts upfield of an aromatic resonance (see Methods). This resonance shifted continuously upfield with each addition of GTP, signifying fast exchange behavior. There were no other perturbations to the NMR spectrum that could be detected in one-dimensional spectra, other than the appearance of peaks from the GTP itself. Two-dimensional TOCSY and NOESY spectra were collected on a sample of HPr with excess GTP in order to identify the perturbed resonance and to ascertain whether additional perturbations had occurred. The resonance in question belongs to Phe48, whose entire spin system was shifted slightly upfield in the presence of GTP. No other chemical shift perturbations were observed anywhere in the TOCSY spectrum. In addition, all carbon-bound proton resonances for GTP could be assigned from the TOCSY spectrum. These were identical to the chemical shifts for free GTP. No intermolecular NOEs were observed for HPr and GTP. The chemical shift of the aromatic resonance of the δ- and ϵ-protons of Phe48 in the S46R mutant of HPr is plotted as a function of added GTP in Figure 8. Both curves suggest a saturation effect. The maximum shift for F48$^{\delta}$H is approximately 0.16 ppm and for F48$^{\epsilon}$H approximately 0.1 ppm.

A 1D NMR GTP titration was repeated with the S46D mutant form of *E. coli* HPr. No spectral perturbations whatsoever were observed, and this observation was confirmed in a 2D NOESY spectrum (data not shown).

DISCUSSION

A previous paper from this laboratory (Peterkofsky & Gollop, 1993) presented a characterization of the response of *E. coli* adenylyl cyclase to nucleotides. It was shown that the ribonucleoside triphosphates (ATP, GTP, CTP, and UTP) were capable of stimulating the activity of the enzyme in a permeable cell system. It was concluded that a single allosteric site responsive to all the RNA precursors plays a role in regulating adenylyl cyclase activity.

In the present work, the results show that the nucleotide stimulation requires the presence of an organized complex of adenylyl cyclase with PTS proteins typical of intact or permeable cells; disruption of the cells is accompanied by a loss of the nucleotide stimulatory effect (Figure 1).

Studies of photoaffinity labeling of PTS proteins with AzGTP (Figure 3) indicated that HPr can specifically bind GTP. Consensus sequences for binding nucleotides to a variety of proteins have been described (Chin et al., 1988; Amin & Peterkofsky, 1992). Such a sequence is (G/A)-XXXX(G/A)K(S/T) (see Figure 9). The sequence of HPr was searched for regions that align with this sequence motif. It was found that the region from amino acids 39 to 46 (GKSASAKS) fit the consensus. It is worth noting that other GTP-binding proteins also contain the nucleotide binding consensus. The bovine p21 ras sequence from residues 10 to 17 is GAGGVGKS (McCaffery et al., 1989); the well-studied sequence of elongation factor EF-Tu from *E. coli* from residues 19 to 26 is GHVDHGKT (An & Friesen, 1980). Least-squares fit of the regions of the crystal structures of the p21 ras and EF-Tu sequences around the nucleotide binding motif region show that they superimpose on each other but not on the HPr sequence. This may partially explain why p21 ras and EF-Tu bind GTP with high affinity but HPr binds the nucleotide with low affinity (see Figures 4 and 8). GTP bound to p21 ras and EF-Tu is considerably less solvent exposed than is the GTP in the model structure with HPr (see below).

The observation (Figure 5) that AzGTP can photolabel HPr from the Gram-negative organism *E. coli* but not the protein from the Gram-positive organism *B. subtilis* or from *M. capricolum* which is evolutionarily related to Gram-positive organisms (Maniloff, 1983) attests to the specificity of the nucleotide interaction with HPr. The sequence alignment shown in Figure 9 of these three HPr proteins indicates that the match to the consensus is four out of four (100%) for *E. coli* HPr, three out of four (75%) for *B. subtilis* HPr, and two out of four (50%) for *M. capricolum* HPr. We therefore propose that this portion of the HPr sequence is an important region for determining the specificity for nucleotide binding to HPr. It should be noted that this portion of the HPr sequence includes serine-46, which is the amino acid residue that is phosphorylated by a metabolite-activated ATP-dependent kinase (Reizer et al., 1984; Reizer & Peterkofsky, 1987) on Gram-positive (*B. subtilis* and *M. capricolum*) but not Gram-negative (*E. coli*) HPr proteins.

FIGURE 9: Sequence comparisons of various HPrs. The sequences in the range of residues 34−54 of the HPr proteins from *E. coli* (Saffen et al., 1987; De Reuse et al., 1985), *B. subtilis* (Gonzy-Treboul et al., 1989), and *M. capricolum* (Zhu et al., 1993) are aligned. Also shown is the consensus for a typical ATP-binding site (Chin et al., 1988). The portion of the *E. coli* sequence in reverse shading corresponds to those positions that were shown to be essential for photolabeling activity. The portions of the *E. coli*, *B. subtilis*, and *M. capricolum* sequences in light shading are totally conserved in the three sequences.

Further evidence for the specificity of the interaction of HPr with GTP comes from the study of a variety of site-directed mutants of *E. coli* HPr (Table 3 and Figures 6 and 7). Consistent with our proposal that the serine-46 region is important for the interaction, it was observed that replacement of this residue with an aspartic acid or glutamic acid residue results in the loss of GTP binding. Interestingly, replacement with the basic amino acid arginine results in a species that binds the photoaffinity ligand more avidly (see Figure 7 and Table 3). It is worth speculating that a positive charge density in the S46 region is required for the interaction with GTP. As discussed below, a model has been formulated that suggests a function for serine-46 in the interaction with GTP. The finding that photolabeling of HPr was retained in the S46R mutant but lost in the S46D mutant of HPr (see Figure 7 and Table 3) was paralleled by NMR spectroscopy experiments (Figure 8) showing GTP-dependent chemical shifts in the wild-type and S46R mutant forms of HPr but not in the S46D mutant.

The concentration of GTP required for maximal stimulation of adenylyl cyclase activity in permeable cells is in excess of 0.7 mM (Peterkofsky & Gollop, 1993). Maximum photoaffinity labeling of HPr by AzGTP requires approximately 1.5 mM nucleotide (Figure 4). Examination of the data (Figure 8) showing GTP-dependent chemical shifts of the F48 resonances indicates that the concentrations of GTP required for maximal chemical shifts are approximately 1 mM for the F48$^{\delta}$H and 0.65 mM for the F48$^{\epsilon}$H. The similarity of these parameters is evidence that the GTP association with HPr might be necessary for the GTP-dependent stimulation of adenylyl cyclase activity.

In a previous study (Peterkofsky & Gollop, 1993), the nucleotide specificity for stimulation of adenylyl cyclase in permeable cells was determined. Stimulation was observed with the nucleoside triphosphates (ATP, GTP, CTP, and UTP) but not with GDP or GMP. The incomplete correlation of the nucleotide specificities for binding to HPr and activation of adenylyl cyclase (five out of seven compounds tested agree) (Figure 4) argues that binding of GTP to HPr may be unrelated to the nucleotide-dependent activation of adenylyl cyclase. However, it is conceivable that the apparent disagreement in specificity patterns might be a reflection of the different test systems used for measuring these parameters.

Molecular modeling studies were carried out to fit GTP into the crystallographic structure of HPr in a manner that would satisfactorily accommodate the data on the binding activity of various HPr mutants (see Figure 10). In this model, the guanine base is wedged in a cavity with Phe48 above and Leu47 and Val23 below the base. Lys24 is near potential hydrogen-bonding groups on the guanine ring. The side chain of Ser46 is proximal to the 3′-OH of GTP. The three phosphate moieties of GTP are close to Lys27, Ser43, Lys45, and Lys49. Although the model suggests a significant number of potential electrostatic interactions between the protein and GTP, the proposed binding site sits on the surface of the protein, which may explain the fairly low apparent binding affinity suggested by the results of this study.

The solution and X-ray structure of HPr indicates an interaction of Ser31 with Asp69 (Hammen et al., 1991). It is possible that the conversion of Asp69 to glutamate might affect the interaction with Ser31 leading to a modification of the binding site region with a resultant effect on the capability of the protein to bind GTP. Alternatively, the Glu69 side chain might exert an electrostatic repulsion effect on the γ-phosphate of GTP leading to a decreased binding of GTP. It has been observed (E. B. Waygood, unpublished experiments) that the D69E form of HPr is unstable and tends toward aggregation. It is possible that there is insufficient space to accommodate Glu69 in a stable structure. Thr36 is located in a region of the protein distant from the nucleotide binding site. The effect of mutation of this residue to Asn with a resultant loss of capability to bind GTP is difficult to explain; perhaps the mutation leads to a disruption of the protein structure.

The data of Figure 7 indicated that mutation of S46 to arginine resulted in a protein with enhanced capability to bind GTP. The model in which GTP was docked to HPr was refit with the S46R mutant. In this case, it appears that the arginine side chain can hydrogen bond with the α-phosphate and a backbone oxygen of GTP. The rearrangement of these side chain interactions might provide an explanation for the increase in avidity of GTP binding by the modified HPr.

The three-dimensional structure of *E. coli* HPr has been deduced by both NMR (Klevit & Waygood, 1986; van Nuland et al., 1994) and X-ray crystallographic (Jia et al., 1993) approaches. The structure has been described to be an open-faced β-sandwich formed by four antiparallel β-strands packed against three α-helices. It is interesting to note that the X-ray crystallographic structure of *Dictyostelium discoideum* nucleoside diphosphate kinase (Dumas et al., 1992) (four antiparallel β-strands packed against four α-helices) is very similar to that of HPr. This enzyme binds the same family of ribonucleoside triphosphates that allosterically activates *E. coli* adenylyl cyclase. It is worth noting that the region of the binding site that interacts with the base portion of nucleotides involves a "sandwich" of the

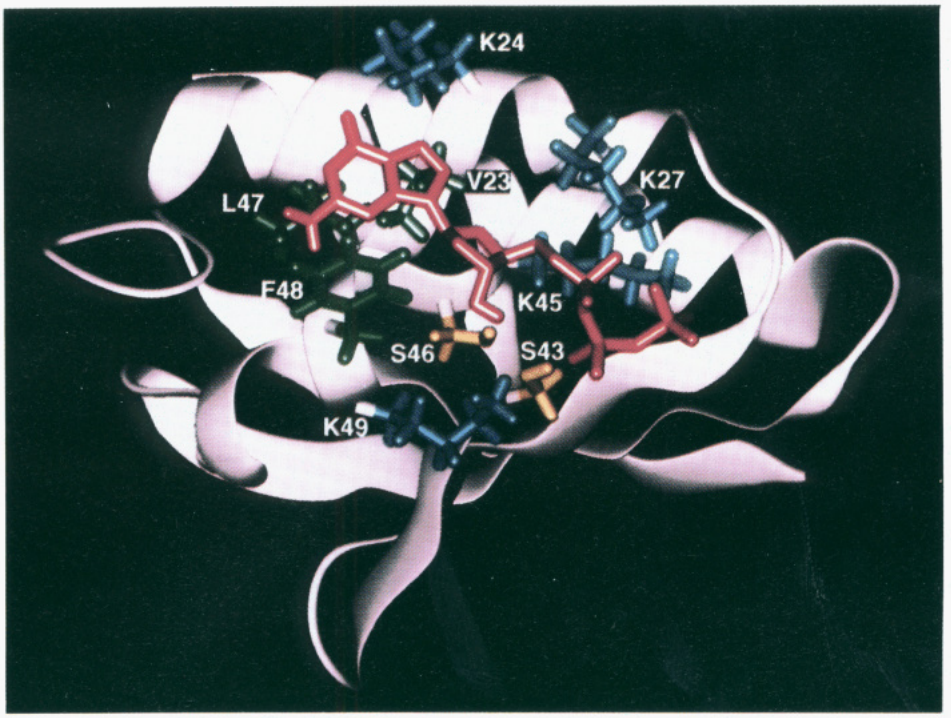

FIGURE 10: Model for interaction of GTP with HPr. The ribbon diagram of HPr was drawn from the crystallographic data using Quanta. The side chains of selected residues are shown in licorice bonds. The molecule of GTP (shown in red) was docked to HPr in the vicinity of the residues shown, and the structure was energy minimized using Charm.

base between a phenylalanine and lysine residue. This is essentially the same arrangement as proposed in our model for the interaction of the guanine base with HPr.

It has been suggested by some workers (Feucht & Saier, 1980; Nelson et al., 1982; Postma et al., 1993) that the regulation of adenylyl cyclase activity by PTS proteins involves an interaction solely with enzyme IIA^{glc}. The previous working model proposed by this laboratory has included not only enzyme IIA^{glc} but also enzyme I and HPr as components of a complex regulating adenylyl cyclase activity. The finding in this study that HPr is a nucleotide-binding protein lends further weight to this idea. The evidence presented in these studies suggesting that GTP binding to HPr is related to the nucleotide-dependent activation of adenylyl cyclase is clearly incomplete. While there are similarities in the concentration dependence profiles and nucleotide specificities, they are not identical. A further test of the relatedness of the two processes might involve an analysis of the effects of site-directed mutants of HPr on the stimulation of adenylyl cyclase activity by GTP. Preliminary attempts to devise a permeable cell system in which the nucleotide-dependent stimulation of adenylyl cyclase is dependent solely on the expression of HPr have been unsuccessful. Further work along these lines or in the direction of a cell-free reconstitution of a GTP-activated adenylyl cyclase will therefore be required to assess the validity of the hypothesis that GTP binding to HPr is physiologically related to the activation of adenylyl cyclase by GTP.

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REFERENCES

Amin, N., & Peterkofsky, A. (1992) *Biochem. Biophys. Res. Commun. 182,* 1218−1225.
An, G., & Friesen, J. D. (1980) *Gene 12*, 33−39.
Anderson, J. W., Bhanot, P., Georges, F., Klevit, R. E., & Waygood, E. B. (1991) *Biochemistry 30*, 9601−9607.
Anderson, J. W., Pullen, K., Georges, F., Klevit, R. E., & Waygood, E. B. (1993) *J. Biol. Chem. 268*, 12325−12333.
Beneski, D. A., Nakazawa, A., Weigel, N., Hartman, P. E., & Roseman, S. (1982) *J. Biol. Chem. 257*, 14492−14498.
Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., & Karplus, M. (1983) *J. Comput. Chem. 4*, 187−217.
Chin, D. T., Goff, S. A., Webster, T., Smith, T., & Goldberg, A. L. (1988) *J. Biol. Chem. 263*, 11718-11728.
De Reuse, H., Roy, A., & Danchin, A. (1985) *Gene 35*, 199−207.
Dumas, C., Lascu, I., Morera, S., Glaser, P., Fourme, R., Wallet, V., Lacombe, M.-L., Veron, M., & Janin, J. (1992) *EMBO J. 11*, 3203−3208.
Feucht, B. U., & Saier, M. H., Jr. (1980) *J. Bacteriol. 141*, 603−610.
Fox, D. K., Presper, K. A., Adhya, S., Roseman, S., & Garges, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7056−7059.
Gonzy-Treboul, G., Zagorec, M., Rain-Guion, M.-C., & Steinmetz, M. (1989) *Mol. Microbiol. 3*, 103−112.
Gottesman, M. M. (1976) *Virology 72*, 33−44.
Grammer, J. C., Kuwayama, H., & Yount, R. G. (1993) *Biochemistry 32*, 5725−5732.
Hammen, P. K., Waygood, E. B., & Klevit, R. E. (1991) *Biochemistry 30,* 11842−11850.
Harwood, J. P., & Peterkofsky, A. (1975) *J. Biol. Chem. 250*, 4656−4662.
Jia, Z., Quail, J. W., Waygood, E. B., & Delbaere, L. T. J. (1993) *J. Biol. Chem. 268*, 22490−22501.
Klevit, R. E., & Waygood, E. B. (1986) *Biochemistry 25*, 7774−7781.
Kundig, W., Ghosh, S., & Roseman, S. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 1067−1074.

Landt, O., Grunert, H.-P., & Hahn, U. (1990) *Gene 96*, 125−128.
Liberman, E., Reddy, P., Gazdar, C., & Peterkofsky, A. (1985) *J. Biol. Chem. 260*, 4075−4081.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
Maniloff, J. (1983) *Annu. Rev. Microbiol. 37*, 477−499.
Mateucci, M. D., & Caruthers, M. H. (1981) *J. Am. Chem. Soc. 103*, 3186−3191.
McCaffery, R. E., Coggins, L. W., Doherty, I., Kennedy, I., O'Prey, M., McColl, L., & Campo, M. S. (1989) *Oncogene 4*, 1441−1448.
Nelson, S. O., Scholte, B. J., & Postma, P. W. (1982) *J. Bacteriol. 150*, 604−615.
Peterkofsky, A. (1977) *Trends Biochem. Sci. 2*, 12−14.
Peterkofsky, A., & Gollop, N. (1993) *Protein Sci. 2*, 498−505.
Peterkofsky, A., Gonzalez, J. E., & Gazdar, C. (1978) *Arch. Biochem. Biophys. 188*, 47−55.
Peterkofsky, A., Reizer, A., Reizer, J., Gollop, N., Zhu, P., & Amin, N. (1993) *Prog. Nucleic Acid Res. Mol. Biol. 44*, 31−65.
Postma, P. W., Lengeler, J. W., & Jacobson, G. R. (1993) *Microbiol. Rev. 57*, 543−594.
Potter, R. L., & Haley, B. E. (1983) *Methods Enzymol. 91*, 613−633.
Reddy, P., Meadow, N., Roseman, S., & Peterkofsky, A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8300−8304.
Reddy, P., Fredd-Kuldell, N., Liberman, E., & Peterkofsky, A. (1991) *Protein Expression Purif. 2*, 179−187.
Reizer, J., & Peterkofsky, A. (1987) in *Sugar Transport and Metabolism in Gram-positive Bacteria*. (Reizer, J., & Peterkofsky, A., Eds.) pp 333−364, Ellis Horwood, Chichester, U.K.
Reizer, J., Novotny, M. J., Hengstenberg, W., & Saier, M. H., Jr. (1984) *J. Bacteriol. 160*, 333−340.
Reizer, J., Sutrina, S. L., Saier, M. H., Jr., Stewart, G. C., Peterkofsky, A., & Reddy, P. (1989) *EMBO J. 8*, 2111−2120.
Reizer, J., Romano, A. H., & Deutscher, J. (1993) *J. Cell. Biochem. 51*, 19−24.
Roy, A., & Danchin, A. (1982) *Mol. Gen. Genet. 188*, 465−471.
Roy, A., Haziza, C., & Danchin, A. (1983) *The EMBO J 2*, 791−797.
Saffen, D. W., Presper, K. A., Doering, T. L., & Roseman, S. (1987) *J. Biol. Chem. 262*, 16241−16253.
Sharma, S. (1992) Ph.D. Thesis, University of Saskatchewan.
Sharma, S., Georges, F., Delbaere, L. T. J., Lee, J. S., Klevit, R. E., & Waygood, E. B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4877−4881.
Sharma, S., Hammen, P. K., Anderson, J. W., Leung, A., Georges, F., Hengstenberg, W., Klevit, R. E., & Waygood, E. B. (1993) *J. Biol. Chem. 268*, 17695−17704.
Stein, J. M., Kornberg, H. L., & Martin, B. R. (1985) *FEBS Lett. 182*, 429−434.
van Nuland, N. A. J., Hangyi, I. W., van Schaik, R. C., Berendsen, H. J. C., van Gunsteren, W. F., Scheek, R. M., & Robillard, G. T. (1994) *J. Mol. Biol. 237*, 544−559.
Vogel H. J., & Bonner D. M. (1956) *J. Biol. Chem. 218*, 97−106.
Waddell, W. J. (1956) *J. Lab. Clin. Med. 48*, 311−314.
Waygood, E. B., Meadow, N. D., & Roseman, S. (1979) *Anal. Biochem. 95*, 293−304.
Zhu, P.-P., Reizer, J., Reizer, A., & Peterkofsky, A. (1993) *J. Biol. Chem. 268*, 26531−26540.

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