NOTES

Modulation of *Escherichia coli* Adenylyl Cyclase Activity by Catalytic-Site Mutants of Protein IIA\textsuperscript{Glc} of the Phosphoenolpyruvate:Sugar Phosphotransferase System

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It is demonstrated here that in *Escherichia coli*, the phosphorylated form of the glucose-specific phosphocarrier protein IIA\textsuperscript{Glc} is an activator of adenylyl cyclase in the toluene-treated cells, the phosphorylated form of the glucose-specific phosphocarrier system is an activator of adenylyl cyclase. To elucidate the specific role of IIA\textsuperscript{Glc} phosphorylation in the regulation of adenylyl cyclase activity, both the phosphorylatable catalytic histidine (H90) and the interactive histidine (H75) of IIA\textsuperscript{Glc} were mutated by site-directed mutagenesis to glutamine and glutamate. Wild-type IIA\textsuperscript{Glc} and the H75Q mutant, in which the histidine in position 75 has been replaced by glutamine, were phosphorylated by the phosphohistidine-containing phosphocarrier protein (HP\textsubscript{r}–P) and were equally potent activators of adenylyl cyclase. Neither the H90Q nor the H90E mutant of IIA\textsuperscript{Glc} was phosphorylated by HP\textsubscript{r}–P, and both failed to activate adenylyl cyclase. Furthermore, replacement of H75 by glutamate inhibited the appearance of a steady-state level of phosphorylation of H90 of this mutant protein by HP\textsubscript{r}–P, yet the H75E mutant of IIA\textsuperscript{Glc} was a partial activator of adenylyl cyclase. The H75E H90A double mutant, which cannot be phosphorylated, did not activate adenylyl cyclase. This suggests that the H75E mutant was transiently phosphorylated by HP\textsubscript{r}–P but the steady-state level of the phosphorylated form of the mutant protein was decreased due to the repulsive forces of the negatively charged glutamate at position 75 in the catalytic pocket. These results are discussed in the context of the proximity of H75 and H90 in the IIA\textsuperscript{Glc} structure and the disposition of the negative charge in the modeled glutamate mutants.

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Adenylyl cyclase catalyzes the synthesis of cyclic AMP (cAMP), which is of central importance in signal transduction, metabolism, and other cellular processes in both eukaryotes and prokaryotes. cAMP levels in the cell are primarily regulated by the modulation of adenyl cyclase activity. Catabolic enzyme synthesis in *Escherichia coli* is regulated by the cellular concentration of cAMP. Despite the vast literature on catabolite repression and the glucose effect that has accumulated over the past couple of decades (12, 14, 29), the precise molecular mechanism for glucose inhibition of cAMP synthesis in *E. coli* remains unclear. Adenylyl cyclase and the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) proteins have been implicated in catabolite repression. Energized by PEP and successively mediated by the PTS proteins, enzyme I, HPr, and enzyme(s) IIABC (25), PTS sugars are translocated into the cell as sugar phosphates. This results in the conversion of the PTS proteins from a phosphorylated state to a dephosphorylated state. Inhibition of adenyl cyclase activity by the PTS sugars has long been known to be due to this event (15). Although genetic evidence suggests that the PTS-catalyzed phosphorylation of IIA\textsuperscript{Glc} is involved in the activation of adenyl cyclase (5, 24), no direct biochemical evidence is yet available to support this model. *E. coli* IIA\textsuperscript{Glc} has two histidines, H75 and H90, in close proximity in the catalytic site (30). Histidine 90 is the target for phosphorylation by the phosphohistidine-containing phosphocarrier protein (HP\textsubscript{r}–P) (4). Histidine 75 is conserved throughout the known proteins IIA\textsuperscript{Glc} of bacterial systems (17). Histidine 75 is required for phosphorylated protein IIA\textsuperscript{Glc} (IIA\textsuperscript{Glc}–P) to act as a phosphate donor to protein IIBC\textsuperscript{Glc} and glucose (18). Based on these biochemical properties and the atomic structures of the IIA\textsuperscript{Glc} family members (10, 30), IIA\textsuperscript{Glc} catalytic-site mutants were created to define the role of IIA\textsuperscript{Glc} phosphorylation in the regulation of adenyl cyclase activity. The results presented here demonstrate that IIA\textsuperscript{Glc}–P is an activator of adenyl cyclase while IIA\textsuperscript{Glc} does not affect the basal enzyme activity.

**DNA manipulations.** Digestion of DNA with restriction enzymes was performed according to the manufacturers’ recommendations. DNA fragments were separated by electrophoresis on SeaKem GTG agarose or NuSieve GTG agarose, and bands were excised and melted at 65°C in an equal volume of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. DNA fragments were purified by phenol extraction and ethanol precipitation. Ligation of DNA fragments was performed as described elsewhere (26). Strain C600 lambda lysogen was used as the host for transformation with the ligation mixtures and for isolation of recombinant plasmids. Competent cells of the *E. coli* strains used here were prepared by the Hanahan method (6).

**Cloning of the crr gene into pACYC184 under the control of P\textsubscript{acg}** In order to express protein IIA\textsuperscript{Glc} at levels as close to the physiological level as possible for studies on the regulation of adenyl cyclase in the toluene-treated cells, the *crr* gene was
cloned into low-copy-number plasmid pACYC184 (3) under the control of Pmcr. The HindIII-Sall fragment of pACYC184 encompassing the tet promoter region and part of the structural gene was cloned into M13mp18. The three bases (5’TGT3’) to the tet initiation codon ATG were mutated to 5’CAT3’ by site-directed mutagenesis (28) to create the NdeI restriction recognition sequence. The HindIII-Sall fragment with the NdeI recognition sequence was amplified by PCR with the M13 forward and reverse primers. The amplified DNA was restricted with HindIII and Sall and cloned back into pACYC184 lacking the same fragment. The NdeI-Sall fragment containing the crr gene was cloned into the newly created Ndel site and the Sall site of pACYC184 such that crr expression would be under the control of Pmcr.

Cloning of the crr gene into the pRE expression vector and purification of protein IIA^Glc^ and the H75Q and H75E mutants. The wild-type and mutant crr structural gene(s) was cloned as NdeI-Sall fragments into the respective sites in the pRE1 expression vector (20). A recombinant containing the wild-type crr gene was introduced into E. coli MZ1 (Xc857) (31). Expression and purification of wild-type protein IIA^Glc^ were accomplished as described previously (21). The mutant proteins were similarly purified after expression in the crr deletion strain TP2865 transformed with plasmid pRK248 carrying the temperature-sensitive repressor (1).

Other methods. The details of the adenyl cyclase assay have been described elsewhere (7). Synthesis of [3^2]PPEP was accomplished as described elsewhere (22). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8), and labeled proteins were detected by autoradiography. Protein was estimated by the Lowry method (11). Oligonucleotides for mutagenesis were synthesized by the phosphoramidite method on an Applied Biosystems 380B DNA synthesizer. DNA sequence was determined, using Sequenase, by the dideoxy method of Sanger et al. (27) as modified by Biggin et al. (2).

Site-directed mutagenesis of histidines in protein IIA^Glc^.

The single-stranded DNA containing the crr gene in M13mp18 with an Ndel site at the initiation codon (21) was the starting material for site-directed mutagenesis of histidines 75 and 90 to glutamine and glutamate at each position. The three-dimensional structure of protein IIA^Glc^ shows that the two histidine residues at positions 75 and 90 form the active site (Fig. 1A) (10, 30). H90 being the target for phosphorylation by HPr-P. It was demonstrated that the H75Q IIA^Glc^ mutant is phosphorylated by HPr-P at H90 but fails to serve a phosphotransfer donor in the subsequent PTS phosphotransfer reaction; thus, it is permanently phosphorylated in the presence of PEP and the general energy coupling proteins enzyme I and HPr (18). In contrast, the H90Q mutant cannot be phosphorylated at all. These mutant proteins allow the examination of whether the phosphorylated or nonphosphorylated IIA^Glc^ regulates adenyl cyclase activity. In addition, the H75E and H90E mutant proteins were analyzed by radioimmunoassay of a negative charge at the active site of this phospocarrier protein may mimic the phosphorylated form of IIA^Glc^.

Regulation of adenyl cyclase activity by the state of phosphorylation of IIA^Glc^.

Strain TP2865, which lacks the crr gene (9), was used to evaluate the ability of the wild-type and mutant IIA^Glc^’s to regulate adenyl cyclase activity. The strain was transformed with plasmid pDIA100, which overproduces adenyl cyclase about 10-fold (23). Strain TP2865/pDIA100 was transformed either with pACYC184 as a control or pACYC184 carrying the wild-type crr gene or with one of the five mutants, H75Q, H75E, H90Q, H90E, and H75E H90A. The results in Table 1 show that the activity of adenyl cyclase, in the presence of potassium phosphate, is strongly stimulated by complementation of the crr deletion strain with wild-type IIA^Glc^ or the H75Q mutant. When the phosphorylation of the catalytic H90 residue is abolished by the H90Q or H90E mutants, adenyl cyclase is not activated. This clearly demonstrates that the activation of adenyl cyclase occurred by virtue of the phosphorylation at H90 of the wild-type and H75Q mutant of IIA^Glc^, because the nonphosphorylatable H90Q mutant failed to activate the enzyme. The H90E mutant also failed to activate adenyl cyclase. However, the ability of H75E IIA^Glc^ to activate adenyl cyclase is intermediate compared to the abilities of the phosphorylated and dephosphorylated forms. This suggests either that the H90 moiety of the H75E IIA^Glc^ mutant is phosphorylated by HPr-P or that the H75E IIA^Glc^ mutant, with the negatively charged glutamate in the catalytic pocket, may mimic the phosphorylated form of the wild-type protein. To address these possibilities, a H75E H90A IIA^Glc^ double mutant was constructed in which phosphorylation at position 90 is prevented. Partial stimulation of adenyl cyclase activity caused by H75E IIA^Glc^ was reversed by the double mutant. This result suggests that H75E IIA^Glc^ may be phosphorylated.

We attribute the level of activation of adenyl cyclase by the IIA^Glc^ mutants to the nature of the mutation because negligible differences in the amount of IIA^Glc^ produced in the pRE1 vector were observed. The amounts of protein(s) produced in the pRE1 vector after 15 min, 30 min, 1 h, and 2 h of P^i induction in E. coli MZ1 were quantitated by gel scanning (data not shown). The amounts of protein(s) IIA^Glc^ produced at each time point were equal, and all of the protein(s) IIA^Glc^ was stable and soluble. Although the protein(s) IIA^Glc^ produced from the pACYC184 vector was not quantitated by gel scanning because of the low level of expression, it is assumed that similar amounts of protein(s) IIA^Glc^ were produced and that the level of activation of adenyl cyclase by the protein(s) IIA^Glc^ was due to the nature of the mutation rather than any differences in the amount of protein(s) IIA^Glc^ produced.

In vitro phosphorylation of IIA^Glc^.

It is interesting to note the major difference between the phosphorylation patterns of the protein IIA^Glc^ H75Q and H75E mutants (Fig. 2). While the
FIG. 1. The active sites of wild-type IIA^{Glc} (A) and modeled H75E (B) and H90E (C) mutants. (A) The proximity of phosphorylatable H90 and the interactive H75 in the catalytic pocket of IIA^{Glc} are shown. It is possible to replace H75 (B) and H90 (C) with glutamate without altering the rest of the structure and yet maintain electrostatic interactions between residues 75 and 90. The figure was produced based on the crystal structure of Bacillus subtilis IIA^{Glc} (10), whose active site is very similar to that of the E. coli protein (30).
glutamine mutant is phosphorylated by HPr→P (lane 3), the glutamate mutant does not appear to be phosphorylated by HPr→P (lane 4). The twofold activation of adenylyl cyclase by H75E IIAGlc may be reconciled by the facts that H75 and H90 in the catalytic pocket are proximal and that the negatively charged glutamate in protein IIAGlc H75E may function like phosphorylated H90 (Fig. 1B). Another plausible explanation for the activation of adenylyl cyclase by the protein IIAGlc H75E mutant is that this mutant gets transiently phosphorylated at H90, resulting in partial activation of adenylyl cyclase, but is rapidly dephosphorylated by the repulsive forces of the adjacent negatively charged glutamate residue such that the mutant is not observed in a steady-state phosphorylated form (Fig. 2, lane 4). A twofold activation of adenylyl cyclase by the H75E IIAGlc mutant and the reversal of this activation by the H75E IIAGlc H90A double mutant favor the argument that a transient phosphorylation of H90 of the H75E mutant may occur. The inability of H90E IIAGlc to activate adenylyl cyclase is not readily understood. Perhaps the carboxyl group of glutamate in the H90E IIAGlc mutant is buried compared to the phosphate group of H90 of the wild-type protein (Fig. 1C).

**Relationship between adenylyl cyclase activation and phosphate pools.** There is substantial evidence that the activation of adenylyl cyclase by potassium phosphate is mediated through the PTS proteins (16). Furthermore, there is a clear relationship between the stimulation of adenylyl cyclase by potassium phosphate and the inhibition of adenylyl cyclase by glucose. Glucose has no effect on adenylyl cyclase basal activity. Only when the basal activity is stimulated by potassium phosphate is inhibition of adenylyl cyclase activity by glucose observed. As expected, glucose or methyl-α-D-glucopyranoside inhibited adenylyl cyclase activity in the strain transformed with the plasmid containing wild-type crr (Table 1). Surprisingly, a very similar result was observed with the H75Q mutant: it has been clearly demonstrated that this mutant cannot be dephosphorylated by methyl-α-D-glucopyranoside when purified PTS proteins are used (18). It is conceivable that in an intact cell, glucose or methyl-α-D-glucopyranoside may be vectorially phosphorylated, albeit at a slow rate, by the mannose pathway. Indeed, the efficient fermentation of glucose as well as mannose by the H75Q mutant (data not shown) is consistent with this interpretation. Thus, in an intact cell with the H75Q mutation, dephosphorylation of HPr can occur, and it in turn can be phosphorylated in the reversible reaction by the phospho-H75Q mutant. Such a turnaround dephosphorylation of the H75Q mutant would result in the deactivation of adenylyl cyclase.

It was shown that addition of glucose to starved *Streptococcus lactis* cells causes a rapid metabolism of and an instantaneous decrease in PEP and inorganic phosphate (P_i) pools from about 40 to 5 mM (13). Since the maximum activation of adenylyl cyclase occurs at 20 mM potassium phosphate, at least one pathway for deactivation of adenylyl cyclase by glucose was suggested to be by decreased cellular P_i pools (16). However, adenylyl cyclase assays were performed in vitro with toluene-treated cells equilibrated with 20 mM potassium phosphate, and addition of 1 mM glucose is not expected to cause any appreciable change in the P_i concentration. Moreover, the pattern of deactivation of adenylyl cyclase with glucose (data not shown) and a nonmetabolizable analog, *methyl*-α-D-glucopyranoside, are indistinguishable. Beyond the phosphorylation of *methyl*-α-D-glucopyranoside at the expense of PEP, no further metabolism takes place to account for the decreased P_i concentration. In fact, *methyl*-α-D-glucopyranoside in nanomolar concentrations has been shown to lower cAMP levels in intact cells of *E. coli* and *Salmonella typhimurium* (5). It appears that the minimum requirements for the high-activity form of adenylyl cyclase are a high P_i concentration and phosphorylated IIAGlc.

**Speculation on the interaction of adenylyl cyclase with protein IIAGlc.** The crystal structure of protein IIA^Glc^ suggests that there may be no gross conformational change in the protein upon phosphorylation (10, 30). Thus, we suggest that the acquisition of a negative charge at H90→P is responsible for the interaction with adenylyl cyclase. Protein IIAGlc→P and adenylyl cyclase may form a stable complex through charge interactions. Protein IIAGlc→P is a positive effector and protein IIAGlc→P is a negative effector of different enzymes. This concerted role of phosphorylated and dephosphorylated protein IIAGlc has been demonstrated in the transport and catabolism of non-PTS sugars like lactose, glycerol, and maltose. While protein IIAGlc acts as a negative effector of the transport systems of these non-PTS sugars by binding to the sugar permeases, protein IIAGlc→P dissociates from the sugar permeases and does not interfere with the transport. Protein IIAGlc→P is a positive effector of adenylyl cyclase and thereby increases levels of cAMP, which is required for the transcription of the catabolic operons of lactose, glycerol, and maltose应该的 cells encounter these sugars.

All the models put forward for the regulation of adenylyl cyclase have been from studies using intact cells (5, 9, 24), toluenized cells (7), or crude extracts (19). Although it is trivial to obtain pure adenylyl cyclase from an overproducing *E. coli* strain (20), the impediment to pinpointing which of the PTS components is responsible for the regulation of adenylyl cyclase activity by the in vitro reconstitution and/or protein-protein interaction has been the failure to obtain the pure enzyme in a regulatable form. It is conceivable that an unidentified factor in the regulation of adenylyl cyclase escaped our search up to now.

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