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The Role of Phosphorylation of HPr, a Phosphocarrier Protein of the Phosphotransferase System, in the Regulation of Carbon Metabolism in Gram-Positive Bacteria

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Abstract HPr of the Gram-positive bacterial phosphotransferase system (PTS) can be phosphorylated by an ATP-dependent protein kinase on a serine residue or by PEP-dependent Enzyme I on a histidyl residue. Both phosphorylation events appear to influence the metabolism of non-PTS carbon sources. Catabolite repression of the gluconate (*gnt*) operon of *B. subtilis* appears to be regulated by the former phosphorylation event, while glycerol kinase appears to be regulated by the latter phosphorylation reaction. The extent of our understanding of these processes will be described.

Key words: phosphotransferase system, HPr, sugar transport, gram-positive bacteria, protein kinase

Anaerobic and facultatively anaerobic eubacteria possess a complex transport system, the phosphoenolpyruvate-dependent phosphotransferase system (PTS) responsible for the uptake of exogenous carbohydrates [Meadow et al., 1990; Reizer et al., 1988a; Saier, 1985; Saier and Reizer, 1992]. A characteristic feature of this system is the tight coupling of sugar transport to phosphorylation. The phosphate group derived from phosphoenolpyruvate is transferred to the incoming sugar via a chain of four phosphorylated protein intermediates (Fig. 1). The high energy phosphoryl groups in most of the PTS phosphoproteins appear to be linked to histidyl residues. In some of these proteins (HPr and some of the Enzymes IIB) the phosphoryl group appears to be linked to the N-1 position of the imidazole ring, but in others (Enzyme I and the Enzymes IIA) the phosphoryl moiety is linked to the proteins at the N-3 position of the imidazole ring. In some Enzymes IIB, the phosphate is probably linked to a cysteyl residue. Sugars transported via the PTS in Gram-positive bacteria include glucose, fructose, mannose, glucosamine, N-acetylglucosamine, mannitol, glucitol, lactose, sucrose, and, in some bacteria, maltose [Reizer et al., 1988a]. As illustrated in Figure 1, Enzyme I and HPr are the general, non-sugar-specific, energy-coupling proteins of the PTS, while the Enzyme II complexes exhibit sugar-specificity. Enzyme I, HPr, and the Enzymes IIA are water-soluble or peripheral membrane proteins while the Enzyme IICs are integral membrane proteins or domains. Each of the polypeptide chains which comprise the Enzyme IICs pass through the lipid bilayer of the membrane at least six times [Saier et al., 1988, 1989].

ATP-DEPENDENT PHOSPHORYLATION OF HPr

In 1983, the ATP-dependent, metabolite-activated phosphorylation of HPr was demonstrated both in vivo [Reizer et al., 1983] and in vitro [Deutscher and Saier, 1983]. The presence of a metabolite-activated protein kinase had been predicted, based on characteristics of the phenomenon of inducer expulsion, a process whereby non-metabolizable cytoplasmic sugar phosphates accumulated via the PTS were rapidly expelled

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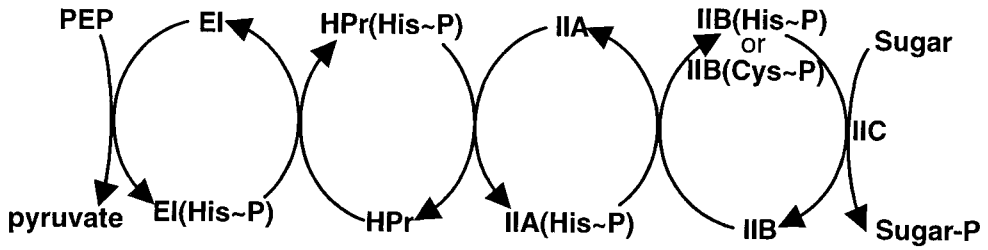

Fig. 1. Schematic representation of phosphoryl transfer reactions catalyzed by the phosphotransferase system. The reaction scheme illustrates the individual phosphoryl transfer reactions and the residues phosphorylated within each protein or domain. Definition and designation of the proteins or domains is as described in Saier and Reizer, 1992.

from the cell as the free sugars upon addition of a metabolizable carbohydrate [Reizer and Panos, 1980; Thompson and Saier, 1981; Reizer et al., 1985]. In vivo experiments had revealed that the process was probably dependent on cytoplasmic ATP and a glycolytic intermediate [for reviews see Reizer, 1989; Reizer and Peterkofsky, 1987]. The early evidence had led to the suggestion that the sugar phosphate phosphatase which cleaves the phosphoryl group from the intracellular sugar phosphates might be activated by protein phosphorylation [Reizer et al., 1983; Thompson and Saier, 1981; Reizer et al., 1985].

Employing the in vivo conditions which gave rise to rapid inducer expulsion in *Streptococcus pyogenes,* a search for stable phosphoproteins revealed the existence of a predominant phosphoprotein of low molecular weight [Reizer et al., 1985]. It did not, however, possess the expected sugar phosphate phosphatase activity, and it was subsequently identified as HPr of the PTS [Deutscher and Saier, 1983]. In addition to HPr from *S. pyogenes,* HPrs from *Enterococcus faecalis, Lactococcus lactis, Streptococcus mutans, Streptococcus salivarius, Staphylococcus aureus, Staphylococcus carnosus, Bacillus subtilis, Bacillus stearothermophilus, Clostridium histolyticum, Lactobacillus casei,* and *Lactobacillus brevis* were all found to undergo ATP-dependent phosphorylation, and all of these organisms possessed the HPr kinase [Reizer et al., 1988a,b; Deutscher and Engelmann, 1984; Reizer et al., 1984a; Waygood et al., 1986; Deutscher et al., 1986; Romano et al., 1987; Mimura et al., 1987]. HPrs from Gram-negative bacteria such as *E. coli* were not phosphorylated by the Gram-positive kinases, and corresponding HPr kinases were not found in these organisms [Deutscher and Engelmann, 1984; Reizer et al., 1984a].

In contrast to the PEP-dependent, Enzyme I-catalyzed phosphorylation of HPr which occurs on a histidyl residue, seryl residue 46 was shown to be modified in the ATP-dependent phosphorylation reaction [Deutscher et al., 1986]. The phosphorylated protein, HPr(Ser-P), was not able to transfer its phosphate group to a sugar [Deutscher and Saier, 1983]. While the region around serine 46 is conserved in all sequenced HPrs of Gram-positive bacteria, HPrs from Gram-negative bacteria showed little sequence identity in this region (Fig. 2).

ATP-dependent HPr kinases which catalyze the formation of HPr(Ser-P) have been partially purified from *E. faecalis, S. pyogenes, Lactobacillus brevis,* and *Bacillus subtilis* [Deutscher and Engelmann, 1984; Reizer et al., 1984a; Romano et al., 1987; Reizer et al., 1988b]. The activities of these enzymes are stimulated by metabolites such as fructose-1,6-diphosphate (FDP), gluconate-6-P, and 2-P-glycerate, and they are inhibited by phosphate [Deutscher and Engelmann, 1984; Reizer et al., 1984a; Romano et al., 1987; Reizer et al., 1988b]. An HPr(Ser-P) phosphatase has also been purified from *E. faecalis* [Deutscher et al., 1985]. The enzyme requires high phosphate (about 50 mM) for optimal activity, but it is not allosterically regulated by glycolytic intermediates.

In this context, the regulation of pyruvate kinase by intracellular metabolites in *L. lactis* [Mason et al., 1981] may be relevant. This enzyme is regulated as are the HPr kinases from *E. faecalis* and *S. pyogenes.* It is stimulated by FDP and inhibited by inorganic phosphate. Investigations on changes in the concentrations of FDP, PEP, and inorganic phosphate which occur when resting cells of *L. lactis* are transferred to a medium containing glucose revealed that the FDP concentration increased to about 25 mM while the concentrations of phosphate and PEP decreased to about 3 mM and 1 mM, respectively [Mason et al., 1981; Thompson and Tor-

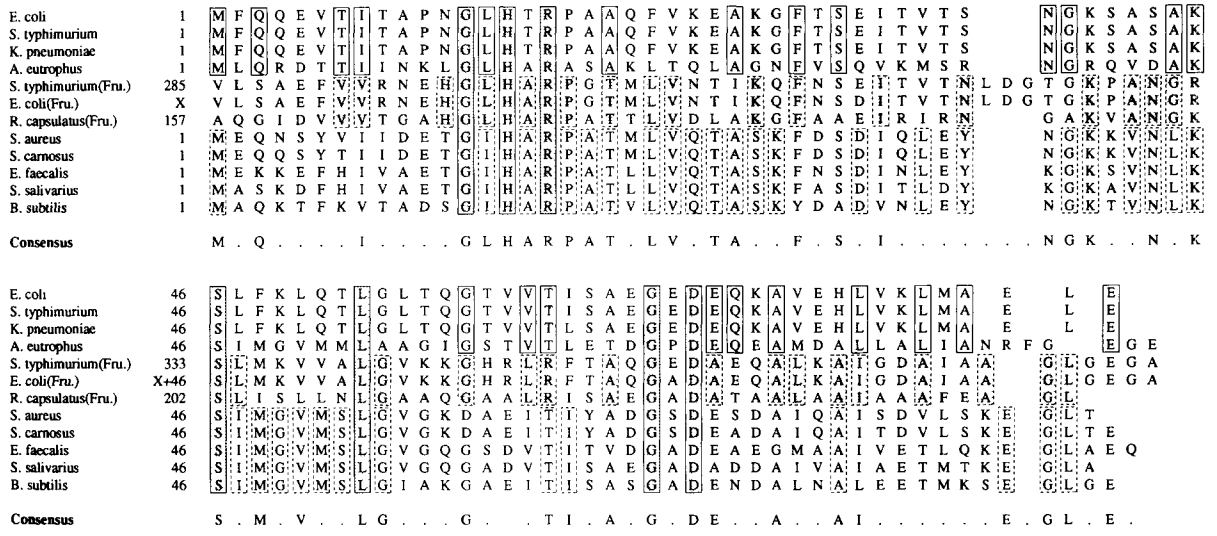

Fig. 2. Alignment of the twelve sequenced HPr proteins or HPr-like protein domains of the bacterial PTS. Boxed and shaded residues indicate amino acids which are conserved in all twelve members of this family. Boxed and unshaded residues indicate amino acids which are conserved in all Gram-negative proteins (not including the fructose inducible protein domains [designated (Fru.)] of the *E. coli* and *S. typhimurium* diphosphoryl transfer protein (DTP), and the *R. capsulatus* multiphosphoryl transfer protein (MTP). Residues conserved in the three fructose-specific protein domains (Fru.) of the *E. coli* and *S. typhimurium* DTP and the *R. capsulatus* MTP are shaded and boxed with dashed lines. Residues conserved in all Gram-positive HPrs are unshaded and boxed with dashed lines. The consensus sequence (Consensus) is shown below the aligned sequences. Numbers indicate the residue number in each of the twelve members of this protein family. Since the complete sequence of the *E. coli* diphosphoryl transfer protein (DTP) is yet to be determined, the first amino acid of its HPr-like protein domain is designated X. Abbreviations and references of published sequences are as follows: *Staphylococcus carnosus* (*S. carnosus;* Eiserman et al., 1991), *Staphylococcus aureus* (*S. aureus;* Reizer et al., 1988a), *Bacillus subtilis* (*B. subtilis;* Reizer et al., 1988; Reizer, 1989; Gonzy-Tréboul, 1989), *Streptococcus salivarius* (*S. salivarius;* Gagnon et al., 1992), *Enterococcus faecalis* (*E. faecalis;* Deutscher et al., 1986), *Salmonella typhimurium* (*S. typhimurium;* Byrne et al., 1988; Powers and Rosemann, 1984), *Escherichia coli* (*E. coli;* DeReuse et al., 1985; Saffen et al., 1987), *Klebsiella pneumonia* (*K. pneumonia;* Titgemeyer et al., 1990), *Alcaligenes eutrophus* (*A. eutrophus;* Pries et al., 1991), *Rhodobacter capsulatus* (Fru.) (*R. capsulatus* (Fru.); Wu et al., 1990), *Salmonella typhimurium* (Fru.) (*S. typhimurium* (Fru.); Geerse et al., 1989), *Escherichia coli* (Fru.) (*E. coli* (Fru.); Orchard and Kornberg, 1990).

chia, 1984]. Similar effects would be expected for other carbohydrates degraded via glycolysis and thus for virtually all PTS sugars.

In a quantitative assay using HPr, HPr-(Ser-P) and Enzyme I from *E. faecalis*, the PEP-dependent phosphorylation of HPr(Ser-P) was shown to be 600-fold slower than the phosphorylation of HPr [Deutscher et al., 1984]. A reciprocal effect of histidyl phosphorylation on the activity of the kinase was also observed [Reizer et al., 1984a, 1989]. Thus, formation of doubly phosphorylated HPr occurs slowly. In the presence of a complete PTS, all kinetic parameters for sugar phosphorylation were defined employing free HPr as well as HPr(Ser-P) [Reizer et al., 1992].

The three-dimensional structure of the *B. subtilis* HPr protein has recently been determined (see Chen et al., this symposium). This small, heat stable protein consists of an open faced β-sandwich formed by four antiparallel β-strands serving as the underlying bread packed against two or three α-helices serving as the overlying spread. As revealed by the X-ray structure, the β-sheet curls back on itself so that the regulatory seryl residue (S46) is close to the active site histidyl residue (H15). Thus, the presence of the negatively charged phosphoryl group at position 46 may inhibit introduction of the phosphoryl group at the active site by electrostatic repulsion or by inhibition of Enzyme I binding. The same explanation applies to inhibition of seryl residue 46 phosphorylation by prior phosphorylation of histidyl residue 15. It should be noted that phosphorylation of seryl residue 46 in HPr and in a site-directed mutant protein which cannot be phosphorylated by Enzyme I and PEP, i.e., H15A, was strongly inhibited by the mere presence of Enzyme I [Reizer et al., 1989]. These results provide compelling evidence that the kinase and Enzyme I share overlapping binding site(s) in HPr. Alternatively, the formation of HPr-Enzyme I complex can induce a conforma-

TABLE I. Catabolite Repression of Gluconate Kinase Synthesis by PTS Sugars in *B. subtilis* Strains*

Sugar(s) present during growth	Gluconate kinase activity		
	GM122	SA003	GM808
None	<1	<1	<1
Gluconate	24 ± 4	24 ± 6	25 ± 4
Gluconate + glucose	2 ± 1	21 ± 6	2 ± 1
Gluconate + mannitol	4 ± 2	20 ± 3	4 ± 2
Gluconate + glucitol	23 ± 3	21 ± 4	24 ± 4

*The three strains used were column 1: GM122 (wild-type); column 2: SA003 (an isogenic mutant in which the regulatory seryl residue in the chromosomally-encoded HPr is replaced by an alanyl residue); column 3: GM808 (a primary site revertant). Enzyme activities are expressed in nmoles of product formed per min per mg protein at 37°C. Experimental conditions will be described in a forthcoming publication (Deutscher et al., in preparation).

tional change in HPr which inhibits the kinase. These two mechanisms are not mutually exclusive.

In vivo studies [Reizer, 1989; Reizer et al., 1989, 1992; Sutrina et al., 1990] did not provide evidence for the suggestion [Deutscher et al., 1984] that seryl phosphorylation of HPr serves as a device for regulating the activity of the PTS. This fact, as well as the recent demonstration of HPr, HPr(Ser) kinase, and HPr(Ser-P) phosphatase in heterofermentative lactobacilli [Romano et al., 1987; Reizer et al., 1988b] and in *Acholeplasma laidlawii* (Reizer et al., in preparation) that lack a functional PTS prompted us to consider an alternative physiological function for the HPr(Ser) kinase-HPr(Ser-P) phosphatase system. As shown in Table I, synthesis of gluconate kinase in a wild-type *Bacillus subtilis* strain is inducible by growth in the presence of gluconate and strongly repressed by inclusion of glucose or mannitol, both PTS sugars in *B. subtilis,* in the growth medium. The non-PTS sugar, glucitol, was not repressive, showing that the repressive phenomenon exhibits specificity for sugar substrates of the PTS. In the second column in Table I, data are presented for an isogenic *B. subtilis* strain, which has a single mutation in the chromosomal structural gene for HPr, a mutation (S46A) which changes the regulatory seryl residue to alanine so that ATP-dependent phosphorylation of this protein cannot occur. The consequence of this mutation is that the repressive effect of the PTS sugars on gluconate kinase synthesis is essentially abolished. When the S46A mutation was reversed (primary site reversion), the wild-type repressive effect was restored, establishing that the mutation was responsible for the observed in vivo response. Very similar behavior was observed for another catabolic enzyme, glucitol dehydrogenase, involved in the catabolism of the non-PTS sugar, glucitol (data not shown).

These results suggest that HPr(Ser) phosphorylation provides a mechanistic basis for one form of catabolite repression [Saier, 1991]. This form of repression clearly differs from the cyclic AMP-dependent mechanism which has been documented in enteric bacteria. It is clear that HPr(Ser) phosphorylation plays a role either in gluconate metabolism or in catabolite repression of the operon encoding the gluconate catabolic enzymes. While the mechanism remains unknown, the involvement of a seryl protein kinase in the phenomenon of catabolite repression is reminiscent of this phenomenon in eukaryotic organisms such as *Saccharomyces cerevisiae* [Saier, 1991].

PEP-DEPENDENT PHOSPHORYLATION OF HPr

In an examination of the phosphorylated proteins of *E. faecalis,* a protein with a molecular weight of about 55,000 was identified which could be phosphorylated in vitro in the presence of Enzyme I and HPr at the expense of [^{32}P]PEP [Deutscher, 1985]. No Enzyme IIA activity was found to be associated with this protein. Attempts to demonstrate a regulatory function for the protein, analogous to that of IIA^{glc} in enteric bacteria, led to its identification as glycerol or dihydroxyacetone kinase [Deutscher and Sauerwald, 1986]. The PTS-catalyzed reversible phosphorylation of this protein was shown to occur at the N-3 position of a histidyl residue and led to a 10-fold increase in activity. In agreement with these biochemical studies, physiological studies have shown that glycerol uptake in *E. faecalis* and *B. subtilis* is regulated by the PTS in a fashion which is explicable by the biochemical observations noted above [Romano et al., 1990; Reizer et al., 1984b]. Thus, glycerol uptake

in these organisms was shown to be inhibited by sugar substrates of the PTS, and a stronger inhibitory effect was exerted by lower concentrations of PTS sugars in Enzyme I deficient mutants than in the isogenic wild-type strains. Furthermore, the concentration of the inhibiting sugar which half maximally blocked glycerol uptake was inversely related to the amount of the accumulated inhibiting carbohydrate [Romano et al., 1990; Reizer et al., 1984b]. Significantly, glycerol kinase activity in *E. faecalis* correlated closely with the glycerol uptake activities described above [Romano et al., 1990].

Using a polyclonal antibody against glycerol kinase, varying amounts of free and phosphorylated glycerol kinase in extracts from cells grown in the presence of different carbon sources were recently demonstrated as follows: glycerol grown cells: a large amount of glycerol kinase was expressed and the majority of the protein (~85%) was identified as the phosphorylated/activated form; cells grown on glucose, fructose or mannitol plus glycerol: much less glycerol kinase was expressed, and a mere 15% of the protein was present in the phosphorylated form (J. Deutscher and B. Bauer, unpublished results). These biochemical data substantiate and are in full agreement with the earlier physiological studies described above [Romano et al., 1990; Reizer et al., 1984b].

The regulation of the HPr(His ~ P)-mediated phosphorylation of glycerol-kinase has been studied [Deutscher and Sauerwald, 1986]. Because glycerol kinase, like IIA^{sugar} proteins of the PTS, was shown to be phosphorylated by HPr(His ~ P) the rate of glycerol kinase phosphorylation was compared with that of IIA^{lac} from *S. aureus*. Phosphorylation of IIA^{lac} by HPr(His ~ P) was approximately 200-fold faster than that of glycerol-kinase. It is noteworthy that the bulk of the HPr in the cultures of *E. faecalis* utilizing a PTS-sugar is in the form of HPr(Ser-P) [Vadeboncoeur et al., 1991]. A valid comparison between phosphorylation of IIA^{lac} and glycerol-kinase should, therefore, employ the doubly phosphorylated phosphocarrier protein, HPr(Ser-P)(His ~ P), rather than HPr(His ~ P). Using HPr(Ser-P)(His ~P), the rate obtained for IIA^{lac} phosphorylation was only twice the rate observed with glycerol-kinase. Surprisingly, HPr(Ser-P)(His ~ P) served as a better phosphoryl donor than HPr(His ~ P) for the phosphorylation of glycerol-kinase. It was proposed that dephosphorylation of phosphoglycerol-kinase is not catalyzed by a protein-phosphate phosphatase but rather occurs by reversal of the phosphorylation reaction, i.e., transfer of the phosphoryl group back to HPr. Taken together, these data support the model that a positive control mechanism, exerted by the PTS, modulates glycerol utilization in *E. faecalis* [Deutscher and Sauerwald, 1986]. According to this scheme, glycerol-kinase can compete with IIA^{sugar} proteins for the phosphoryl groups coming from HPr(His ~ P) or HPr(Ser-P)(His ~ P). In the absence of sugar substrates of the PTS, glycerol-kinase can be activated due to phosphorylation of the enzyme by HPr(His ~ P). On the other hand, addition of a PTS carbohydrate results in rapid dephosphorylation of the PTS proteins, which might promote effective phosphoryl group transfer from the phosphorylated glycerol-kinase to HPr or HPr(Ser-P). Consequently, the unphosphorylated, less active form of glycerol-kinase should predominate in cells metabolizing a PTS sugar. This mode of regulation of glycerol kinase activity clearly explains the repressive effect of PTS sugars on glycerol kinase synthesis (see above). Accordingly, the reduced activity of glycerol kinase, due to depletion of the phosphate group from HPr(His ~ P) following addition of a PTS sugar, can limit the intracellular concentration of glycerol phosphate, which, in turn, serves as the inducer of glycerol kinase synthesis as it does in *E. coli* [Hayashi and Lin, 1965]. Thus, in contrast to the apparent negative control exerted by the PTS on glycerol dissimilation in enteric bacteria (see Saier's contribution to this symposium), a positive mode of PTS-mediated regulation appears to function in *E. faecalis* [Deutscher and Sauerwald, 1986; Romano et al., 1990].

OVERVIEW OF TWO DISTINCT MECHANISMS OF CATABOLITE REPRESSION IN GRAM-POSITIVE BACTERIA

As presented above, the available evidence suggests that two different operons (*gnt* and *glp*) in two different organisms (*B. subtilis* and *E. faecalis*) are subject to catabolite repression by two distinct mechanisms, both of which involve HPr of the PTS. Both of these mechanisms are mediated by HPr, but different residues in this protein are phosphorylated to give rise to the repressive phenomenon. In *B. subtilis*, ATP-dependent seryl phosphorylation appears to be involved in *gnt* operon repression, but in *E. faecalis*, PEP-dependent histidyl phosphorylation, which results in direct phosphorylation of the key inducer generating enzyme, glyc-

erol kinase, abolishes catabolite repression. Thus, the former mechanism may correspond to negative control while the latter mechanism is clearly a positive control mechanism. The latter type of catabolite repression results from negation of this positive control mechanism when the presence of a PTS sugar drains phosphate off the PTS-phosphorylated glycerol kinase. This event in turn reduces the generation of cytoplasmic inducer, thus decreasing the extent of operon expression. These two dissimilar mechanisms thereby achieve the same goal, namely, the construction of a heirarchy of preferred sugars for growth with PTS sugars at the top of the heirarchy.

Recent studies have shown that expression of the *B. subtilis glpD* gene, encoding glycerol-3-phosphate dehydrogenase, is regulated by antitermination [Holmberg and Rutberg, 1991]. Since antitermination of the β-glucoside (*bgl*) operon of *E. coli* and the sucrose (*sac*) system of *B. subtilis* are regulated by PTS-mediated phosphorylation (see A. Wright's contribution to this symposium), it must be considered that similar mechanisms will be found for the *glp* operon of *B. subtilis* and other Gram-positive bacteria. Thus, the PTS may exert control over the utilization of a non-PTS carbohydrate at several levels. Further studies will be required to establish the implications of these preliminary observations.

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