REVIEW ARTICLE

Catabolite repression and inducer control in Gram-positive bacteria

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Overview

Over the past quarter of a century, tremendous effort has allowed elucidation of crucial aspects of the mechanisms of catabolite repression and cytoplasmic inducer control in *Escherichia coli* and other Gram-negative enteric bacteria (Botsford & Harman, 1992; Postma *et al.*, 1993; Saier, 1989, 1993; Saier *et al.*, 1996). The extent of this effort reflected in part the belief that 'What is true for *E. coli* is also true for elephants' (J. Monod), and that the mechanism observed for *E. coli* would therefore prove to be universal. The observation that catabolite repression and inducer exclusion (Magasanik, 1970) are universal phenomena, documented in phylogenetically distant bacteria as well as in eukaryotes, seemed to substantiate this suggestion (Saier, 1991). Early investigations consequently focused on *E. coli* for a detailed understanding of the mechanisms involved.

More recently, several research groups have begun to examine the mechanisms controlling carbohydrate catabolism in bacteria other than *E. coli*. In most cases, clear mechanistic concepts have not yet crystallized. However, in one group of prokaryotes, the low-GC Gram-positive bacteria, crucial aspects of the underlying mechanism are emerging. The proposed mechanism involves the proteins of the phosphoenolpyruvate (PEP)-dependent sugar transporting phosphotransferase system (PTS) as in *E. coli*, but the proteins that are directly involved in regulation and the mechanisms responsible for this control are completely different. This review provides a synopsis of recent advances concerned with the details of this process.

The bacterial phosphotransferase system catalyses the concomitant uptake and phosphorylation (group translocation) of its sugar substrates (PTS sugars) via the PTS phosphoryl transfer chain as follows:

Virtually all low-GC Gram-positive bacteria that have been examined, including species of *Bacillus*, *Staphylococcus*,

Streptococcus, *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Mycoplasma*, *Acholeplasma*, *Clostridium* and *Listeria*, possess the enzymes of the PTS. In select Gram-positive bacteria, glucose has been shown to repress synthesis of both PTS and non-PTS carbohydrate catabolic enzymes; it also inhibits the uptake of both PTS and non-PTS sugars (inducer exclusion) while stimulating dephosphorylation of intracellular sugar-Ps and/or efflux of the free sugars (inducer expulsion) (Fig. 1). Substantial evidence supports the contention that a metabolite-activated ATP-dependent protein kinase phosphorylates a seryl residue in HPr to regulate enzyme synthesis, inducer exclusion and inducer expulsion. A single allosteric regulatory mechanism acting on different target proteins is probably involved (Deutscher *et al.*, 1994, 1995; Saier & Reizer, 1994; Ye *et al.*, 1994a–d, 1996; Ye & Saier, 1995a, b, c; Saier *et al.*, 1995a, b).

ATP-dependent protein kinases have long been recognized as important regulatory catalysts for the control of cellular catabolic, anabolic, and differentiative processes in higher organisms (Fischer, 1993; Krebs, 1993). For example, cAMP-dependent protein kinases regulate the rates of glycogen, lipid, and amino acid catabolism in animals, and these kinases as well as cAMP-independent protein kinases influence the phenomenon of catabolite repression in yeast (Wills, 1990; Saier, 1991). An involvement of protein kinase cascades in the control of eukaryotic gene transcription and cellular growth is also well established (Davis, 1993; Neiman, 1993; Blumer & Johnson, 1994).

The two best-characterized target substrates of bacterial protein kinases are isocitrate dehydrogenase in enteric bacteria and HPr of the PTS in Gram-positive bacteria (Deutscher & Saier, 1988). Phosphorylation of the former protein controls the relative activities of the Krebs cycle and the glyoxylate shunt while phosphorylation of the latter protein apparently controls the initiation of carbohydrate metabolism at both protein and gene levels. Interestingly, both the isocitrate dehydrogenase kinases of enteric bacteria and the HPr kinases of Gram-positive

Fig. 1. Proposed functions of HPr(ser)-phosphorylation by the ATP-dependent metabolite-activated HPr(ser) kinase in low-GC Gram-positive bacteria. Abbreviations: HPr, heat-stable phosphocarrier protein of the phosphotransferase system (PTS); PK, HPr(ser) kinase; Pase, HPr(ser-P) phosphatase; +, activation; −, inhibition; CRE, catabolite-responsive element. Inducer exclusion by inhibition of the PTS has been documented in *L. lactis* and *B. subtilis*; inducer expulsion by activation of a sugar-P phosphohydrolase (Pase II) has been documented in *L. lactis*, *Strep. bovis*, *Strep. pyogenes* and *Ent. faecalis*; inducer exclusion and expulsion by uncoupling H⁺ symport from sugar transport via non-PTS transport systems has been documented in *Lact. brevis*; catabolite repression by enhancing the binding of repressor proteins CcpA (and possibly CcpB) to the control regions of catabolite-sensitive operons has been documented in *B. subtilis*.

bacteria appear to differ from eukaryotic protein kinases in recognizing the tertiary structures rather than the primary structures of their target proteins (Saier, 1994). A primary role of protein phosphorylation in the regulation of carbon and energy metabolism in all major classifications of living organisms is suggested (Saier & Chin, 1990; Cozzone, 1993; Saier, 1993).

In this review we will discuss how the PTS transports its sugar substrates (PTS sugars) and concomitantly represses synthesis of carbohydrate catabolic enzymes, inhibits uptake of PTS and non-PTS sugars and/or stimulates efflux of accumulated PTS and non-PTS sugars in a species-specific fashion. We shall see that the metabolite-activated, HPr kinase mediates these regulatory mechanisms by phosphorylating Ser-46 in HPr. HPr(ser-P) exhibits low activity as a phosphoryl carrier protein, accounting for inhibition of PTS sugar uptake, and it binds to and allosterically regulates the activities of various non-PTS transport proteins, hydrolytic enzymes and transcription factors. We summarize the recent advances that have led to an understanding of the molecular details of these related regulatory phenomena and point out the differences and parallels with corresponding processes in Gram-negative enteric bacteria such as *E. coli* and *Salmonella typhimurium*.

Early description of sugar expulsion in streptococci and lactococci

Sugar uptake in bacteria is regulated by a variety of mechanisms defined previously (Saier, 1985, 1989, 1993; Saier *et al.*, 1995a, b). One such regulatory device, a vectorial process which controls sugar or sugar phosphate accumulation by efflux of the intracellular sugar from the cell, occurs in Gram-positive bacteria (Reizer *et al.*, 1985, 1988a, 1993a, b). Expulsion of intracellular sugars was

first observed during studies on transport of β -galactosides in resting cells of *Streptococcus pyogenes* and *Lactococcus lactis* (Reizer & Panos, 1980; Thompson & Saier, 1981). Like many other lactic acid bacteria, starved cultures of *Strep. pyogenes* and *L. lactis* use the PTS and cytoplasmic stores of PEP for uptake and phosphorylation of PTS sugars such as lactose and its non-metabolizable analogue, methyl- β -D-thiogalactopyranoside (TMG). Subsequent addition of a metabolizable PTS sugar (but not a non-metabolizable sugar) to a bacterial culture preloaded with TMG elicits rapid dephosphorylation of intracellular TMG-phosphate and efflux of the free sugar. The half-time for expulsion of TMG by glucose (15–20 s) is much shorter than that for accumulation of the sugar phosphate. Since pulse labelling experiments revealed that the intracellular pool of TMG-P is essentially stable in the absence of glucose, expulsion of TMG is not due to rapid turnover of TMG-P and inhibition of TMG influx by glucose. A cytoplasmic sugar-P phosphatase is apparently activated, and rapid, energy-independent efflux of the sugar follows (Reizer *et al.*, 1983, 1985; Sutrina *et al.*, 1988).

Discovery of HPr(ser-P) in Gram-positive bacteria

Attempts to define the mechanism of sugar expulsion led to the identification of a unique phosphorylated derivative of HPr. A small protein was initially shown to be phosphorylated in cultures of *Strep. pyogenes* in response to conditions which promote expulsion (Reizer *et al.*, 1983). In further studies, the low molecular mass protein was phosphorylated *in vitro* employing a crude extract of *Strep. pyogenes* in the presence of [γ -³²P]ATP, and after purification, the isolated protein was identified as a phosphorylated derivative of HPr (Deutscher & Saier, 1983). This phosphorylated HPr differed from HPr(his-15 ~ P), the

high-energy phosphoprotein that promotes sugar transport and phosphorylation. The novel HPr-derivative proved to be HPr(ser-46-P) (Deutscher & Saier, 1983; Reizer *et al.*, 1984, 1989a; Deutscher *et al.*, 1986).

The streptococcal HPr(ser) kinase has a molecular mass of approximately 60 000 Da, and its activity is dependent on divalent cations as well as on one of several intermediary metabolites, the most active of which are fructose 1,6-bisphosphate (FBP) and gluconate 6-P (Gnt 6-P) (Reizer *et al.*, 1984; Deutscher & Engelmann, 1984). Phosphorylation of the seryl residue in HPr is strongly inhibited by inorganic phosphate or by phosphorylation of the active-site histidyl residue (Reizer *et al.*, 1984). The converse is also observed, i.e. Ser-46 phosphorylation strongly inhibits His-15 phosphorylation. This last observation suggested a potential mechanism for regulation of sugar uptake via the PTS (Deutscher *et al.*, 1984).

Since the discovery of the HPr kinase in *Strep. pyogenes*, protein kinases which phosphorylate HPr have been described in work from several laboratories for a number of low-GC (but not high-GC) Gram-positive bacteria (see Reizer *et al.*, 1988b, for an early review; Hoischen *et al.*, 1993; Titgemeyer *et al.*, 1994, 1995). The phosphorylation of Ser-46 in HPr has been implicated in the regulation of PTS activity on the basis of *in vitro* studies (Deutscher *et al.*, 1984), but the physiological significance of this observation was until recently questioned, due to limited *in vivo* studies conducted with *Bacillus subtilis* and *Staphylococcus aureus* (Reizer *et al.*, 1989a; Sutrina *et al.*, 1990; Deutscher *et al.*, 1994; see below). The kinetic parameters influenced by HPr(ser) phosphorylation have been defined in *in vitro* PTS-catalysed sugar phosphorylation reactions (Reizer *et al.*, 1989a, 1992). The development of methods for the *in vivo* quantification of the four forms of HPr [free HPr, HPr(ser-P), HPr(his-P), and HPr(ser-P, his-P)] have resulted in the unexpected finding that substantial amounts of the doubly phosphorylated form of HPr exist in streptococci (Vadeboncoeur *et al.*, 1991). The strong inhibition of HPr(his) phosphorylation via the Enzyme I-catalysed reaction by phosphorylation of Ser-46 has been reported to be relieved by *in vitro* complexation of HPr with an Enzyme IIA such as the IIA protein specific for gluconate (Deutscher *et al.*, 1984), but the significance of this observation has been questioned (Reizer *et al.*, 1989a, 1992).

Overproduction, purification and properties of site-specific HPr mutants of *B. subtilis*

To determine the significance of HPr(ser) phosphorylation to the regulation of physiological processes, site-specific mutants were constructed in which Ser-46 in the *B. subtilis* HPr was replaced by alanine (S46A) or aspartate (S46D). Similarly, His-15 of this phosphocarrier protein was replaced with alanine (H15A) or glutamate (H15E) (Eisermann *et al.*, 1988; Reizer *et al.*, 1989a; unpublished results). These proteins were overproduced and purified. About 20 mg pure protein (1 culture medium)⁻¹ was obtained with each protein when cells were grown to early stationary-phase. The proteins were characterized

with respect to their catalytic functions, and the consequences of the presence of the mutant genes were determined in *B. subtilis* strains that were deleted for the chromosomal HPr gene (*ptsH*) (Reizer *et al.*, 1989a, b, 1992). *Staph. aureus ptsH* and *ptsI* mutants were also investigated, and when the various HPr-encoding plasmids were transferred into *Staph. aureus*, they behaved similarly to those present in analogous *B. subtilis* strains. Enzyme I and PEP phosphorylated S46D HPr exceedingly slowly due to the negative charge at position 46 of this protein (Reizer *et al.*, 1992).

In accordance with the *in vitro* observations, bacterial strains bearing S46A HPr were shown to ferment PTS sugars nearly as well as the wild-type bacteria, but strains bearing S46D HPr fermented PTS sugars poorly. Transport studies revealed that the rates and extent of fructose, glucose, mannitol, and maltose uptake by the S46D HPr-bearing strains were strongly reduced. The kinetic constants for *in vitro* sugar phosphorylation revealed that the Enzyme-I-catalysed phosphorylation of HPr had a 20-fold higher K_m for the S46D mutant HPr than for the wild-type protein with a V_{max} that was about 20% lower (Reizer *et al.*, 1992). These results substantiated the view that phosphorylation of Ser-46 in HPr has the potential to regulate sugar uptake via the PTS *in vivo* provided that most of the cellular HPr becomes modified by seryl phosphorylation.

Involvement of HPr(ser-P) in the regulation of the PTS and a cytoplasmic sugar-P phosphatase in *L. lactis* and other low-GC Gram-positive bacteria

Both inhibition of PTS sugar uptake and stimulation of sugar-P release from the cytoplasmic compartments of several low-GC Gram-positive bacteria have recently been shown to be dependent on HPr(ser-P) (Ye *et al.*, 1994b, d, 1996; unpublished results). These regulatory processes are depicted schematically in Fig. 2. In wild-type *B. subtilis*, uptake of a PTS sugar such as [¹⁴C]fructose or [¹⁴C]mannitol proved to be strongly inhibited by the presence of a high concentration of extracellular glucose. However, in the chromosomal *ptsH1* mutant in which wild-type HPr is replaced by the S46A mutant HPr (a derivative that cannot be phosphorylated by the metabolite-activated ATP-dependent kinase), little or no inhibition was observed. Moreover, electroporation of FBP and HPr into *L. lactis* vesicles strongly depressed the initial rates of uptake of TMG, lactose, and other PTS sugars (unpublished results). These results clearly suggest that phosphorylation of Ser-46 in HPr exerts an inhibitory effect on the PTS *in vivo* under conditions where uptake of a PTS sugar is measured in the presence of excess glucose. Metabolite activation of the HPr(ser) kinase therefore provides an indirect mechanism for the feedback regulation of PTS-mediated sugar uptake. As the PTS initiates the glycolytic pathway in these organisms, this mechanism represents a key regulatory process controlling sugar metabolism via glycolysis (Fig. 2).

Early studies established that expulsion of PTS-accumulated sugar phosphates from *Strep. pyogenes* or *L. lactis*

Fig. 2. Proposed mechanisms for the inhibition of PTS-dependent sugar uptake (inducer exclusion) and the activation of sugar-P phosphatase II (Pase II)-dependent sugar-P hydrolysis which is followed by sugar efflux (inducer expulsion) mediated by phosphorylation of HPr on Ser-46. Abbreviations are as described in the legend to Fig. 1 with the following additional abbreviations: PEP, phosphoenolpyruvate; I, Enzyme I of the PTS; HPr(his-P), the histidyl-phosphorylated form of HPr involved in sugar transport; HPr(ser-P), the seryl-phosphorylated form of HPr involved in regulation; IIABC, a sugar permease (Enzyme II complex) of the PTS; Pase II, the small, heat-stable HPr(ser-P)-activated sugar-P phosphatase found in several low-GC Gram-positive bacteria. Cytoplasmic sugar-P feeds into the glycolytic sugar catabolic pathway, and hence the regulatory interactions depicted represent the first step in the regulation of glycolysis.

occurs in a two step process with cytoplasmic sugar-P hydrolysis being rate-limiting and preceding efflux (see above). It was also shown that in toluidenized vesicles, TMG-P hydrolysis is stimulated by S46D HPr or by wild-type HPr under conditions that activate the HPr(ser) kinase (Ye *et al.*, 1994b). These observations suggested that activation of a sugar-P phosphatase should be demonstrable *in vitro* as well as *in vivo*.

In 1983, Thompson & Chassy had purified a hexose-6-P phosphohydrolase (designated phosphatase I, or Pase I) from *L. lactis*, but they did not provide evidence for or against its potential activation by HPr(ser-P). Following purification of this enzyme to near-homogeneity, Ye & Saier (1995c) showed that this enzyme was not subject to activation by S46D HPr, and it exhibited a substrate specificity that differed from that of the HPr(ser-P)-activated phosphatase. Moreover, it was strongly inhibited by fluoride, a property not shared by the HPr(ser-P)-activated enzyme. These observations clearly suggested that HPr(ser-P) activates a phosphatase that is distinct from the one characterized by Thompson & Chassy (1983).

Activation of a peripherally membrane-associated sugar-P phosphatase (designated phosphatase II or Pase II) by S46D HPr in crude extracts of *L. lactis* was initially demonstrated using any one of several sugar-P substrates. The enzyme was solubilized from the membrane using a mixture of 8 M urea and 4 % (v/v) butanol and purified to apparent homogeneity (Ye & Saier, 1995c). It proved to be a small (9000 Da), heat-stable (100 °C) protein with unusual characteristics. For example, it exhibited broad specificity for sugar-P substrates and was not inhibited by conventional phosphatase inhibitors such as fluoride. Its activity was stimulated over 10-fold by HPr(ser-P) or S46D HPr. Wild-type HPr or the S46A mutant HPr derivative was not stimulatory. Moreover, chemical

crosslinking experiments established that the monomeric enzyme bound S46D HPr with 1:1 stoichiometry. Wild-type HPr and S46A HPr did not inhibit complex formation between the phosphatase and S46D HPr suggesting that the former proteins do not exhibit appreciable affinity for the enzyme. It seems clear that this newly identified enzyme is the one which initiates metabolite-activated, HPr(ser) kinase-dependent inducer expulsion in *L. lactis*.

Several, but not all, low-GC Gram-positive bacteria exhibit the inducer expulsion phenomenon. With this foreknowledge, the occurrence of the small S46D HPr-stimulatable Pase II was investigated in several representative species. As summarized in Table 1, Pase II was found in all bacteria that exhibit the sugar-P hydrolysis-dependent expulsion phenomenon but not in those that do not (Cook *et al.*, 1995a, b; Ye *et al.*, 1996). Thus, the enzyme was found in *L. lactis*, *Strep. pyogenes*, *Streptococcus bovis* and *Enterococcus faecalis*, bacteria that exhibit inducer expulsion, but not in *Streptococcus mutans*, *Streptococcus salivarius*, *Lactobacillus brevis*, *B. subtilis* or *Staph. aureus*, bacteria that do not (Table 1). These observations provide correlative support for the conclusion that this small enzyme initiates the inducer expulsion phenomenon whenever the 'inducer' is a sugar-P that accumulates in the cytoplasm as a result of the activity of the PTS (Fig. 2).

Once the cytoplasmic sugar-P is hydrolysed to free sugar and inorganic phosphate, the sugar rapidly exits the cell by an energy-independent mechanism. The transport system that is responsible was shown to exhibit low affinity for intracellular sugar substrates ($K_m > 10$ mM) and a high temperature coefficient ($Q_{10} = 3.0$, with a calculated activation energy of 23 kcal mol⁻¹/96.6 kJ mol⁻¹; Reizer *et al.*, 1983; Sutrina *et al.*, 1988). These are characteristics that might be expected for a facilitative carrier. Some data were interpreted to suggest that the

Table 1. Correlation of the occurrence of sugar-P hydrolysis-dependent inducer expulsion in cells with the presence of S46D-stimulated Pase II in various low-GC Gram-positive bacteria

Organism	Presence of HPr and HPr(ser) kinase	Occurrence of inducer expulsion	Presence of HPr(ser-P)-stimulated Pase II
<i>L. lactis</i>	+	+	+
<i>Strep. pyogenes</i>	+	+	+
<i>Strep. bovis</i>	+	+	+
<i>Ent. faecalis</i>	+	+	+
<i>Lact. brevis</i>	+	—*	—
<i>Staph. aureus</i>	+	—	—
<i>B. subtilis</i>	+	—	—
<i>Strep. mutans</i>	+	—	—
<i>Strep. salivarius</i>	+	—	—

* In *Lact. brevis*, inducer expulsion of free sugar results from the uncoupling of sugar transport from H⁺ cotransport (see text). This mechanism, though mediated by HPr(ser-P), is distinct from the sugar-P hydrolysis-dependent expulsion mechanism summarized here that is required when the sugars are accumulated as the phosphate esters via the PTS.

PTS Enzymes II might provide the pathway for sugar efflux (Reizer *et al.*, 1983).

A low affinity, high capacity glucose facilitator has been characterized in *Strep. bovis* (Russell, 1990). This bacterium exhibits biphasic kinetics of glucose uptake when the sugar uptake rate is plotted versus the glucose concentration. High-affinity uptake is due to the PTS while low-affinity uptake is due to the facilitator. The latter system is apparently not dependent on ATP hydrolysis or the proton-motive force (Russell, 1990). It is possible that this facilitator mediates inducer expulsion following hydrolysis of cytoplasmic sugar phosphates.

Recently a similar sugar facilitator has been identified in a *ptsI* mutant of *Ent. faecalis* that lacks Enzyme I of the PTS (Romano *et al.*, 1990; unpublished results). The facilitator resembles the low-affinity transporter from *Strep. bovis* in that it apparently functions by an energy-independent mechanism and exhibits low affinity for glucose (in the low mM range). Uptake of glucose or TMG is subject to inhibition by several structurally dissimilar sugars and sugar analogues, suggesting that the system exhibits broad substrate specificity. Preliminary results suggest that the system is present in a broad range of low-GC Gram-positive bacteria (unpublished results). Further experiments will be required to determine if this system is solely or partially responsible for the rapid efflux of sugar during the inducer expulsion process.

Involvement of HPr(ser-P) in the regulation of non-PTS permeases in *Lact. brevis*

In contrast to homofermentative lactic acid bacteria (discussed above) which transport most sugars via the PTS, heterofermentative lactobacilli such as *Lact. brevis* transport glucose, lactose, and their non-metabolizable

analogues, 2DG and TMG, respectively, by active transport energized by the proton-motive force (Romano *et al.*, 1979, 1987). In these bacteria, only fructose is taken up via the PTS (Saier *et al.*, 1996). *Lact. brevis* possesses an ATP-dependent HPr kinase, and it exhibits metabolite-activated, vectorial sugar expulsion by a mechanism that does not depend on sugar-P hydrolysis (Reizer *et al.*, 1988a, b). Thus, [¹⁴C]TMG accumulates in the cytoplasm of lactobacilli when provided with an exogenous source of energy such as arginine, but addition of glucose to the preloaded cells promotes rapid efflux that establishes a low cellular concentration of the galactoside. Counterflow experiments have shown that metabolism of glucose by *Lact. brevis* apparently results in the conversion of the active β -galactoside transport system to a facilitated diffusion system (i.e. from a sugar:H⁺ symporter to a sugar uniporter; Romano *et al.*, 1987, confirmed in Ye *et al.*, 1994a). It appears that HPr(ser-P) binds to the cytoplasmic surface of the lactose permease to uncouple sugar transport from H⁺ cotransport (Fig. 3). The fact that HPr(ser-P)-mediated regulation of cytoplasmic inducer levels occurs regardless of whether uptake occurs via the PTS or a non-PTS permease is particularly worthy of note.

Employing electroporation to transfer purified proteins and membrane impermeant metabolites into right-side-out vesicles of *Lact. brevis*, pre-accumulated free (non-phosphorylated) TMG was shown to efflux from the vesicles upon addition of glucose if and only if intravesicular wild-type HPr was present (Ye *et al.*, 1994a). Glucose could be replaced by intravesicular (but not extravesicular) FBP, Gnt 6-P, or 2-phosphoglycerate, but not by other phosphorylated metabolites, in agreement with the allosteric activating effects of these compounds on HPr(ser) kinase measured *in vitro* (Reizer *et al.*, 1984).

Fig. 3. Proposed mechanism for the regulation of sugar permease function by the PTS in *Lact. brevis*. The lactose: H^+ symport permease or the glucose: H^+ symport permease of *Lact. brevis* transports its sugar substrates (S) by secondary active transport. Proton cotransport allows coupling of sugar uptake to the proton-motive force. The presence of a metabolite, fructose 1,6-bisphosphate (FBP) or gluconate 6-phosphate (Gnt 6-P), activates the HPr(ser) kinase to phosphorylate HPr on Ser-46 (HPr-P). HPr-P binds to target permeases to uncouple sugar transport from proton transport. The permeases then catalyse facilitated diffusion and cannot accumulate their substrates against a concentration gradient.

Intravesicular S46D HPr promoted regulation, even in the absence of glucose or a metabolite. HPr(ser-P) converted the vesicular lactose/ H^+ symporter into a sugar uniporter (Ye *et al.*, 1994a). Glucose metabolism had previously been shown to cause similar uncoupling of sugar transport from proton symport when intact cells were examined (Romano *et al.*, 1987). Moreover, 2DG uptake and efflux via the glucose: H^+ symporter of *Lact. brevis* were shown to be similarly regulated (Ye *et al.*, 1994c). Uptake of the natural, metabolizable substrates of the lactose, glucose, mannose, and ribose permeases was shown to be inhibited by wild-type HPr in the presence of FBP or by S46D HPr in similar vesicle preparations. These last observations clearly suggest metabolic signifi-

cance in addition to the significance of this regulatory mechanism to the control of inducer levels. Thus, intracellular metabolites activate the kinase to provide a feedback mechanism to limit the overall rate of sugar uptake. The regulatory mechanism is apparently not merely designed to create a hierarchy of preferred sugars.

In recent experiments, the direct binding of ^{125}I -labelled [S46D]HPr to *Lact. brevis* membranes containing high levels of the lactose permease (Ye & Saier, 1995a) or the glucose permease (Ye & Saier, 1995b) was demonstrated. The radioactive protein was found to bind to membranes prepared from galactose-grown *Lact. brevis* cells in the presence (but not in the absence) of one of the substrates of the lactose permease. Membranes from glucose-grown cells did not exhibit lactose analogue-promoted ^{125}I -labelled [S46D]HPr binding, but binding was observed in the presence of glucose or 2DG. The substrate and inducer specificities for binding correlated with those of the two permeases for transport. The apparent sugar binding affinities calculated from the sugar-promoted ^{125}I -labelled [S46D]HPr binding curves were in agreement with sugar transport K_m values. Moreover, [S46D]HPr but not wild-type or [S46A]HPr effectively competed with ^{125}I -labelled [S46D]HPr for binding (Ye & Saier, 1995b). These results suggest that free HPr and S46A HPr do not bind to the permeases and that seryl phosphorylation is required to observe an interaction. They establish the involvement of HPr(ser-P) in the proposed regulatory mechanism for PTS-mediated control of non-PTS permeases and suggest a direct, allosteric binding mechanism. The details of the proposed mechanism are illustrated in Fig. 3.

Involvement of HPr(ser-P) in catabolite repression in *B. subtilis*

Catabolite-repressed genes in *B. subtilis* are controlled by more than one global regulatory mechanism. Although none of the *Bacillus* catabolite repression mechanisms is understood at the molecular level, it is clear that they are not analogous to the cAMP-CRP-dependent mechanism that is operative in *E. coli* (Fisher, 1987; Klier & Rapoport, 1988; Fisher & Sonenshein, 1991; Saier, 1991; Rygus & Hillen, 1992; Sizemore *et al.*, 1992; Chambliss, 1993; Stewart, 1993; Deutscher *et al.*, 1994; Hueck & Hillen, 1995; Saier *et al.*, 1995a, b).

Recent publications suggest that the ATP-dependent phosphorylation of Ser-46 in HPr plays a key role in catabolite repression of several catabolic operons in *B. subtilis* (Saier *et al.*, 1992; Deutscher *et al.*, 1994, 1995; Fujita *et al.*, 1995; Ramseier *et al.*, 1995b; unpublished results). This discovery was preceded by the work of Fujita and coworkers who showed that the gluconate (*gnt*) operon is subject to a cAMP-independent catabolite repression mechanism which is dependent on a catabolite-responsive element (CRE) sequence in the *gnt* promoter region and requires metabolism of the repressing sugar (Nihashi & Fujita, 1984; Miwa & Fujita, 1990, 1993; Reizer *et al.*, 1991; Hueck *et al.*, 1994). The metabolite directly causing repression seemed to be an early, common

intermediate of glycolysis since mutations abolishing phosphoglucose isomerase activity abolished repression by exogenous glucose, but loss of glycerol-P dehydrogenase or phosphoglycerate kinase activity did not (Nihashi & Fujita, 1984). Inducer exclusion and expulsion of [^{14}C]gluconate were not demonstrably operative in *B. subtilis* suggesting that the observed repression was not due to these phenomena (Deutscher *et al.*, 1994; Fujita & Miwa, 1994).

Since the HPr(ser) kinases in *B. subtilis* and other Gram-positive bacteria are allosterically activated by intermediates of glycolysis, the most effective of which is FBP, it was possible that HPr phosphorylation mediates catabolite repression. In considering this possibility, several target enzymes in *B. subtilis* were examined (Deutscher *et al.*, 1994). For this purpose, the wild-type chromosomal *ptsH* gene was replaced by the S46A mutant *ptsH* gene so that the mutant HPr protein product was expressed at the same level as its wild-type counterpart but could not be phosphorylated by the HPr(ser) kinase. This mutant, designated *ptsH1*, exhibited nearly wild-type rates of PTS sugar uptake (Reizer *et al.*, 1989a, b, 1992; Deutscher *et al.*, 1994), but synthesis of the gluconate, glucitol and mannitol catabolic enzymes was completely resistant to catabolite repression (Deutscher *et al.*, 1994). Glucose-elicited catabolite repression was restored when the genomic S46A *ptsH* gene was again replaced by the wild-type *ptsH* gene. Inositol dehydrogenase exhibited partial sensitivity to catabolite repression in the S46A HPr mutant, but glycerol kinase and α -glucosidase exhibited normal sensitivity.

Sensitivities of the various operons to HPr(ser-P)-dependent catabolite repression, defined using the *ptsH1* mutant strain, correlated with the dependency of catabolite repression on the CcpA transcription factor, a conclusion based on studies with a *B. subtilis* *ccpA* mutant (Deutscher *et al.*, 1994). CcpA is believed to act generally through the CRE DNA sequence to mediate catabolite repression of some operons and catabolite activation of other operons concerned with carbon metabolism (Grundy *et al.*, 1993, 1994; Hueck *et al.*, 1994; Hueck & Hillen, 1995; Kim *et al.*, 1995; Ramseier *et al.*, 1995b). CcpA is homologous to members of the LacI–GalR family (Vartak *et al.*, 1991; Weickert & Adhya, 1992; Nguyen & Saier, 1996). The CRE sequence to which the protein binds conforms to a palindromic consensus sequence similar to those recognized by several members of the LacI–GalR family. One of the latter proteins is the catabolite repressor/activator (Cra) protein (formerly designated FruR) of enteric bacteria (Ramseier *et al.*, 1993, 1995a; Cortay *et al.*, 1994). Cra mediates a cAMP-independent form of catabolite repression in these organisms.

To further substantiate the involvement of HPr(ser) phosphorylation in catabolite repression in *B. subtilis*, a chromosomal *ptsHI* deletion mutant was isolated and transformed with plasmids carrying the wild-type or mutant *ptsH* genes. The strain synthesizing the S46D mutant HPr exhibited three- to fivefold lower activities of

target enzymes than those synthesizing wild-type or S46A HPr, most likely due to a repressing effect of the S46D HPr on synthesis. These observations strongly suggest that phosphorylation of Ser-46 in wild-type *B. subtilis* HPr by the ATP-dependent HPr kinase or replacement of Ser-46 in HPr with a negatively charged residue promotes catabolite repression.

A plausible catabolite repression mechanism, consistent with these results and illustrated in Fig. 4, is as follows: (1) glucose, or another rapidly metabolizable carbohydrate, generates metabolic intermediates such as FBP via glycolysis or Gnt 6-P via the Entner–Doudoroff or pentose phosphate pathway; (2) the HPr(ser) kinase is activated and phosphorylates HPr on Ser-46; (3) HPr(ser-P), possibly together with a cytoplasmic metabolite, binds to and activates the CcpA protein, inducing a conformation that possesses high affinity for the CRE in the regulatory regions of catabolite-sensitive operons (Hueck *et al.*, 1994); (4) this nucleoprotein complex, possibly together with other effector molecules, retards or blocks transcriptional initiation for catabolite-repressible operons but activates glucose-inducible operons (Grundy *et al.*, 1993, 1994; Deutscher *et al.*, 1994, 1995; Fujita *et al.*, 1995; Ramseier *et al.*, 1995b).

Strong evidence for this proposal has recently been obtained by showing that HPr(ser-P) and S46D HPr, but not free HPr, can bind to the CcpA protein *in vitro*. The CcpA protein used in these studies bore an N-terminal His-tag that allowed it to be immobilized on a Ni^{2+} column (Deutscher *et al.*, 1995; unpublished results). This interaction between CcpA and HPr(ser-P) was reported to be dependent on high concentrations of FBP. Surprisingly, replacement of His-15 with another amino acid residue, or phosphorylation of His-15 with phosphoenolpyruvate and Enzyme I of the PTS, was observed to prevent binding of the protein to CcpA (Deutscher *et al.*, 1995; unpublished results). These surprising results suggest that catabolite repression is determined by a dual phosphorylation mechanism that renders the phenomenon responsive to both intracellular metabolite and extracellular PTS sugar concentrations.

In vitro DNA-binding experiments conducted in three different laboratories with three different operons have yielded somewhat different results. Kim *et al.* (1995) noted that the purified *B. subtilis* CcpA protein binds specifically and with high affinity to the CRE in the *amyO* control region in the absence of HPr(ser-P). In this study the effect of HPr(ser-P) was not examined. Ramseier *et al.* (1995b) used *Bacillus megaterium* CcpA with an N-terminal His-tag, purified by Ni^{2+} -affinity chromatography, to measure the binding interactions of the protein to the CRE of the *xyl* operon of *B. subtilis*. Like Kim *et al.* (1995), they observed DNA–CcpA binding at low protein concentrations. They further showed that HPr or the S46A mutant HPr had no effect on the binding reaction, but that HPr(ser-P) or the S46D mutant HPr interacted with CcpA to diminish the extent of binding to the DNA. Finally, Fujita *et al.* (1995) examined binding of CcpA to the control region of the *gnt* operon of *B. subtilis* and observed that HPr(ser-P) enhanced

Fig. 4. Proposed mechanism for the regulation of the transcription of catabolite-sensitive operons in *B. subtilis*. The metabolite-activated HPr(ser) kinase phosphorylates Ser-46 in HPr, converting it to a form that can bind to the transcription factor, CcpA. These two proteins, possibly together with a metabolite such as fructose 1,6-bisphosphate (FBP), form a complex which binds to CREs in or near the promoter regions of catabolite-sensitive target operons to promote catabolite repression.

binding of CcpA to the CRE in this operon. Whether the differences reported in the three laboratories represent differences in experimental conditions or physiologically relevant differences due to the different systems studied has yet to be determined.

Recent work has provided evidence for a second DNA-binding protein in *B. subtilis* that is apparently involved in catabolite repression (unpublished results). The catabolite control protein B (CcpB), like CcpA, is a member of the LacI-GalR family of transcription factors with an N-terminal helix-turn-helix DNA-binding domain. CcpB exhibits 30% sequence identity with CcpA. Greatest sequence identity between these two proteins was observed in the DNA binding domains. When *B. subtilis* cultures were grown in liquid medium with high agitation, CcpA proved to be the sole mediator of catabolite repression. However, when the same bacteria were grown in liquid medium with low agitation, or when grown on solid agar plates, CcpA and CcpB proved to contribute equally to the intensity of catabolite repression. It appears that these two putative transcription factors function in parallel, both in response to HPr(ser-P) concentrations, to allow the catabolite repression phenomenon to be sensitive to environmental conditions (unpublished results). These exciting preliminary findings suggest the existence of a previously unrecognized degree of complexity in the catabolite repression process. The details of this complex mode of regulation should prove most interesting.

3-Dimensional structural analyses of HPr, HPr(ser-P) and its mutant derivatives

High resolution structural data are available for *B. subtilis* HPr, its seryl phosphorylated form and several of its mutant derivatives (Wittekind *et al.*, 1989, 1990; Kapadia *et al.*, 1990; Herzberg *et al.*, 1992). HPr resembles a skewed open-faced β -sandwich with four antiparallel β -strands underlying three α -helices (Fig. 5). The active site His-15 residue and the regulatory Ser-46 residue are not in direct contact with each other but are also not distant from each other. Phosphorylation of S46 was initially suggested to induce a local conformational change which was believed to be transmitted partly through secondary structural elements to the active site H15 residue, thereby providing an explanation for the reciprocal inhibitory effect of Ser-46 phosphorylation on the PTS phosphoryl transfer reaction (Reizer *et al.*, 1989a, 1994; Wittekind *et al.*, 1989, 1990). Electrostatic and steric effects were also proposed to be important (Wittekind *et al.*, 1989, 1990, 1992).

Recently, Pullen *et al.* (1995) used multidimensional NMR to further define the effects of seryl phosphorylation on the structure and stability of *B. subtilis* HPr. Phosphorylation of Ser-46 was found to stabilize a short helix (helix-B) that exhibits behaviour indicative of conformational averaging in unphosphorylated HPr in solution. Backbone amide proton exchange rates of helix-B residues

Fig. 5. 3-Dimensional structure of *B. subtilis* HPr. This single-domain protein exhibits the structure of an open-faced sandwich with four β -strands comprising a skewed β -sheet (forming the bread of the sandwich) and three overlying α -helices (forming the spread of the sandwich). His-15 (H15), the catalytic phosphorylation site residue involved in PTS-mediated sugar uptake and phosphorylation, is localized to the loop between the first β -strand and the first α -helix (helix A) while Ser-46 (S46), the regulatory site residue involved in all PTS-mediated regulatory functions, is localized to the loop between the third β -strand and the second α -helix (helix B). Phosphorylation of Ser-46 is believed to extend and stabilize helix B. N, N-terminus; C, C-terminus.

were shown to decrease following seryl phosphorylation. Phosphorylation apparently stabilizes the protein to solvent and thermal denaturation, with a ΔG of 0.7–0.8 kcal mol⁻¹ (2.94–3.36 kJ mol⁻¹). A similar stabilization was measured for S46D HPr, indicating that an electrostatic interaction between the negatively charged groups and the N-terminal end of the helix contributes significantly to the stabilization. The results were interpreted to suggest that phosphorylation of Ser-46 at the N-terminal end of α -helix B does not cause a conformational change *per se* but rather stabilizes the helical structure. These observations may have long range relevance to other protein targets of protein kinase-catalysed phosphorylation both in bacteria and in eukaryotes.

In addition to the aforementioned studies on HPr, X-ray diffraction and NMR studies have provided detailed structural data for the *B. subtilis* IIA^{Glc} protein (Fairbrother *et al.*, 1991, 1992a, b; Liao *et al.*, 1991; Stone *et al.*, 1992). Most recently, the intermolecular binding interfaces of HPr and IIA^{Glc} were defined (Chen *et al.*, 1993). The nature of the HPr–IIA^{Glc} interaction may prove relevant to those for the interactions of HPr(ser-P) with its various target permeases, enzymes and transcription factors. Although the latter interactions have not yet been defined, the structural basis for an in-depth understanding of HPr(ser-P)-mediated regulatory phenomena seems to be just around the corner.

Identification of PTS proteins and PTS-mediated regulatory systems in other Gram-positive bacteria

Novel PTSs or PTS proteins have recently been identified in several Gram-positive bacteria. For example: (1) a fructose-specific PTS and a metabolite-activated HPr(ser) kinase/phosphatase are present in *Listeria monocytogenes* (Mitchell *et al.*, 1993); (2) *Acholeplasma laidlawii* possesses Enzyme I and HPr as well as an unusual metabolite-inhibitable HPr(ser) kinase/phosphatase system (Hoischen *et al.*, 1993); (3) the rumen bacterium *Strep. bovis* has been shown to exhibit the phenomenon of inducer exclusion/expulsion mediated by the HPr(ser) phosphorylation mechanism but with some very interesting variations on the recognized theme (Cook *et al.*, 1995a, b); (4) *Lact. brevis* has been found to specifically induce the synthesis of a fructose-specific PTS during anaerobic growth in the presence of fructose (Saier *et al.*, 1996); (5) three species of *Streptomyces* were shown to possess complete fructose-specific PTSs but no detectable HPr(ser) kinase (Titgemeyer *et al.*, 1994, 1995); (6) finally, complete PTSs specific for a variety of sugars have been identified in *Arthrobacter ureafaciens*, *Corynebacterium glutamicum*, and *Brevibacterium helvolum* (V. P. Juban, L. F. Wu, J. Reizer, C. Blanco & M. H. Saier, Jr, unpublished results) although *in vitro* assays have failed to reveal the presence of an HPr(ser) kinase. Thus, while the PTS seems to be present in both high and low-GC Gram-positive bacteria, the HPr(ser) kinase is apparently restricted to the low-GC organisms. It can be anticipated that in addition to its role in the initiation of sugar metabolism, the PTS will prove to play regulatory roles in most, if not all of these bacteria. Multiple complex mechanisms are likely to be involved.

Inducer expulsion phenomena, probably related to HPr(ser) phosphorylation, have been characterized in (1) *Strep. pyogenes*, (2) *Strep. bovis*, (3) *Ent. faecalis*, (4) *L. lactis* and (5) *Lact. brevis*. However, extensive studies to identify comparable phenomena in (1) *B. subtilis*, (2) *Staph. aureus*, (3) *Strep. mutans* and (4) *Strep. salivarius* have yielded consistently negative results (Saier & Simoni, 1976; Deutscher *et al.*, 1994; Ye *et al.*, 1996; unpublished results). In *B. subtilis*, HPr(ser) phosphorylation apparently mediates catabolite repression and regulation of sugar uptake via the PTS, but it does not mediate inducer expulsion. In *Lact. brevis*, inducer exclusion and expulsion result from the control of non-PTS permeases rather than of the PTS and the HPr(ser-P)-activated sugar-P phosphatase. We therefore believe that the specific physiological targets, and hence the consequences of HPr(ser) phosphorylation are different in different low-GC Gram-positive genera.

Little evidence is available to suggest whether or not HPr(ser-P) mediates catabolite repression in Gram-positive bacteria other than species of *Bacillus* and *Staphylococcus*. However, the presence of CREs in the regulatory regions of many other Gram-positive bacterial catabolic operons (Hueck *et al.*, 1994; Hueck & Hillen, 1995) leads

Table 2. Sensory transduction elements in PTS regulatory mechanisms of Gram-negative and Gram-positive bacteria

	Transduction element in:	
	Gram-negative bacteria	Gram-positive bacteria
Compound sensed	Extracellular sugar	Intracellular metabolites
Receptor	Sugar-recognition site of Enzyme IIC	Allosteric site of HPr(ser) kinase
Signal transmission	Phosphoryl transfer from IIA ^{Glc} ~ P via IIB/HPr	Phosphoryl transfer from ATP via HPr(ser) kinase
Effector protein	IIA ^{Glc}	HPr
Consequence of signal	Histidyl dephosphorylation	Seryl phosphorylation
Reversal of signal	Enzyme I/HPr-catalysed rephosphorylation	Protein-P-phosphatase-catalysed dephosphorylation
Known targets	Lactose:H ⁺ symporter	Lactose:H ⁺ symporter
	Glycerol kinase	Sugar-P phosphatase
	Adenylate cyclase	Catabolite repression (CcpA)

to the suspicion that this type of PTS-mediated regulation is widespread in low-GC Gram-positive bacteria. Further studies will be required to establish the range of regulatory targets of HPr(ser) phosphorylation present in these and other Gram-positive bacteria.

Conclusions and perspectives

Results currently available clearly indicate that the metabolite-activated protein kinase-mediated phosphorylation of Ser-46 in HPr plays a key role in catabolite repression and the control of inducer levels in low-GC Gram-positive bacteria. This protein kinase is not found in enteric bacteria such as *E. coli* and *Salmonella typhimurium* where an entirely different PTS-mediated regulatory mechanism is responsible for catabolite repression and inducer concentration control. In Table 2 these two mechanistically dissimilar but functionally related processes are compared (Saier *et al.*, 1995b). In Gram-negative enteric bacteria, an external sugar is sensed by the sugar-recognition constituent of an Enzyme II complex of the PTS (IIC), and a dephosphorylating signal is transmitted via the Enzyme IIB/HPr proteins to the central regulatory protein, IIA^{Glc}. Targets regulated include (1) permeases specific for lactose, maltose, melibiose and raffinose, (2) catabolic enzymes such as glycerol kinase that generate cytoplasmic inducers, and (3) the cAMP biosynthetic enzyme, adenylate cyclase that mediates catabolite repression (Saier, 1989, 1993). In low-GC Gram-positive bacteria, cytoplasmic phosphorylated sugar metabolites are sensed by the HPr kinase which is allosterically activated. HPr becomes phosphorylated on Ser-46, and this phosphorylated derivative regulates the activities of its target proteins. These targets include (1) the PTS, (2) non-PTS permeases (both of which are inhibited) and (3) a cytoplasmic sugar-P phosphatase which is activated to reduce cytoplasmic inducer levels. Other important tar-

gets of HPr(ser-P) action are (4) the CcpA protein and probably (5) the CcpB transcription factor. These two proteins together are believed to determine the intensity of catabolite repression. Their relative importance depends on physiological conditions. Both proteins may respond to the cytoplasmic concentration of HPr(ser-P) and appropriate metabolites. CcpA possibly binds sugar metabolites such as FBP as well as HPr(ser-P). Because HPr(his-P, ser-P) does not bind to CcpA, the regulatory cascade is also sensitive to the external PTS sugar concentration. Mutational analyses (unpublished results) suggest that CcpA may bind to a site that includes His-15. Interestingly, both the CcpA protein in the Gram-positive bacterium, *B. subtilis*, and glycerol kinase in the Gram-negative bacterium, *E. coli*, sense both a PTS protein and a cytoplasmic metabolic intermediate. The same may be true of target permeases and enzymes in both types of organisms, but this possibility has not yet been tested.

The parallels between the Gram-negative and Gram-positive bacterial regulatory systems are superficial at the mechanistic level but fundamental at the functional level. Thus, the PTS participates in regulation in both cases, and phosphorylation of its protein constituents plays key roles. However, the stimuli sensed, the transmission mechanisms, the central PTS regulatory proteins that effect allosteric regulation, and some of the target proteins are completely different. It seems clear that these two transmission mechanisms evolved independently. They provide a prime example of functional convergence.

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