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ATP-Dependent Protein Kinase Activities in the Oral Pathogen *Streptococcus mutans*

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ATP-dependent protein kinase activities were detected in both membrane and cytoplasmic fractions from the oral pathogen *Streptococcus mutans*. Different polypeptides were phosphorylated by endogenous kinase(s) in the two fractions. In membranes, five phosphoproteins were detected with apparent masses of 82, 37, 22, 12, and 10 kilodaltons (KD). In cytoplasm, two major acid-stable phosphoproteins were found. One was identified as HPr of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), while the other had an apparent mass of 61 KD. Both of these proteins were phosphorylated on a seryl residue. Fructose 1,6-bisphosphate stimulated phosphorylation of HPr by the kinase and inhibited phosphorylation of the 61-KD protein. In contrast, fructose 1-phosphate, 2-phosphoglycerate, 3-phosphoglycerate, and dihydroxyacetone phosphate inhibited phosphorylation of HPr and stimulated phosphorylation of the 61-KD protein. Several other glycolytic intermediates as well as inorganic phosphate inhibited phosphorylation of either or both proteins. Preincubation of cytoplasm with PEP prior to incubation with ATP reduced the amount of phospho-(seryl)-HPr formed, but not that of the 61-KD phosphoprotein. The latter protein has not yet been identified but has properties that suggest that it may be the protein kinase itself. These results provide evidence for one or more soluble ATP-dependent protein kinases in *S mutans* that are regulated by glycolytic intermediates and that may play a role in the modulation of carbohydrate uptake and metabolism in this organism. A model for feedback regulation of sugar transport in *S mutans*, mediated by an allosterically regulated kinase, is presented.

Key words: protein phosphorylation, bacterial protein kinases, bacterial phosphotransferase system, HRr of *Streptococcus mutans*, sugar transport, glycolytic regulation

Abbreviations used: PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; HPr, heat-stable phosphocarrier protein of the PTS; TDM buffer, buffer containing 20 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, and 10 mM $MgCl_2$; PEP, phosphoenolpyruvate; Fru-P_2, fructose 1,6-bisphosphate; F1P, fructose 1-phosphate; 2PG and 3PG, 2- and 3-phosphoglycerate, respectively; KD, kilodaltons.

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Recently, reversible protein phosphorylations dependent on ATP and catalyzed by protein kinases and phosphatases, a phenomenon long known to be of regulatory significance in eukaryotic organisms [1], have been demonstrated in both gram-negative [2,3] and gram-positive [4] bacterial species. At least two kinase-phosphorylated proteins have been identified as to function in *Escherichia coli*, isocitrate dehydrogenase [5,6] and RNA polymerase [7], and one such protein has been identified in several gram-positive species as a heat-stable phosphocarrier protein, HPr, of the bacterial sugar phosphotransferase system [4,8,9]. Protein kinases have been purified from *E coli* [7,10], from *Streptococcus faecalis* [11] and have been partially purified from *Streptococcus pyogenes* [8]. Phosphorylation of *E coli* isocitrate dehydrogenase [5,6] and of gram-positive HPrs [8,12] has been shown to be of potential regulatory significance in vivo.

Our laboratory has been studying the mechanism and regulation of sugar uptake in the oral pathogen *Streptococcus mutans*. This organism is believed to be the primary etiological agent of dental caries in man and other animals by virtue of its acidogenic fermentation of dietary sugars resulting in demineralization of tooth surfaces (for a review, see [13]). It seemed likely that *S mutans* would also possess ATP-dependent protein kinases, and in this report we give evidence for such activities in both membrane and cytoplasmic cell fractions. We have identified one kinase-phosphorylated protein as HPr of the PEP-dependent sugar PTS, and we present results that suggest a role for protein kinases in regulating carbohydrate metabolism in *S mutans*.

METHODS

Materials

[γ-^{32}P]ATP, [^{14}C]sucrose, and [^{14}C]glucose were obtained from New England Nuclear Corp. Other reagents were purchased from Sigma Chemical Co.

Bacterial Growth and Cell Lysis

S mutans strain V843 (GS-5, serotype c) was obtained from Dr. F. Macrina. It was routinely cultured on a complex medium containing 2% sucrose and was lysed and fractionated as previously described [14]. Cytoplasmic fractions were concentrated tenfold [14] and dialyzed against 200 volumes of TDM buffer with one change to remove small molecules.

Assays

ATP-dependent protein kinase activities in cell-free fractions were assayed as follows. Each reaction mixture (40 μl) contained membrane or cytoplasmic fractions (20 μl) plus 0.1 mM [γ-^{32}P]ATP, 1 mM KF, 50 mM Tris/HCl (pH 7.5), and other additions as described in the figure legends and table footnotes. Mixtures were incubated at 37°C for 5 min, boiled for 3 min in 125 mM Tris/HCl (pH 6.8), 4% sodium dodecylsulfate, 20% glycerol, 10% 2-mercaptoethanol, 0.3% Bromphenol Blue, and then applied to a dodecylsulfate polyacrylamide gel as described [15]. Gels were stained with Coomassie Brilliant Blue, dried, and exposed to Kodak X-OMAT X-ray film at 4°C. Molecular weights of labeled bands were deduced from standard proteins run in parallel.

PTS enzymes were assayed using membrane and cytoplasmic fractions as described [14].

For identification of phosphoamino acids, polyacrylamide gel pieces containing labeled proteins were excised from dried gels, rehydrated with TDM buffer, and thoroughly minced. These gel fragments were incubated with 100 μl TDM buffer with shaking at 37°C for 30 min. The entire suspension was hydrolyzed by the addition of HCl to 6 N and incubation at 110°C for 4 hr. The hydrolysate was lyophilized, resuspended in 10 μl H_20, and 3 μl of this was applied to a 0.1 mm cellulose sheet (Brinkmann Instruments). Electrophoresis was then performed at 1,000 V for 2 hr in glacial acetic acid:formic acid:H_20 (78:25:897, vol/vol/vol), pH 1.9 [4]. Separated phosphoamino acid standards (1 μg each) run in parallel were visualized with ninhydrin in 0.2% acetone, and [^{32}P]phospho-amino acids were detected by autoradiography.

Antibody Preparation and Immunoblotting

Antibody against HPr was prepared using HPr purified as described previously [14]. Then 0.5 ml (0.3 mg/ml) of pure HPr in TDM buffer was emulsified with an equal volume of Freund's complete adjuvant and was injected intradermally at several locations on a New Zealand white rabbit. The animal was boosted at 3 weeks and again at 4 weeks postchallenge with identical samples except that incomplete adjuvant was used. Blood was collected every 7–10 days thereafter. IgG was purified from both preimmune and immune serum as described by Vadeboncoeur [16] and was stored at −70°C at a concentration of approximately 6 mg/ml. Immunoblotting of dodecylsulfate polyacrylamide gels with purified IgG fractions was performed as previously described [17].

RESULTS

Protein Kinase Activities in *S mutans*

As shown in Figure 1, phosphorylation of discrete polypeptides in both membrane and cytoplasmic fractions of *S mutans* could be detected after incubation of these fractions with [γ-^{32}P]ATP. In membranes, five bands were resolved in autoradiograms of dodecylsulfate polyacrylamide gels of the labeled membrane fraction (Fig. 1, lane A). These polypeptides had apparent masses in KD of 82, 37, 22, 12, and 10. In the cytoplasmic fraction, two prominently labeled bands were resolved with apparent masses of 61 and 15 KD (Fig. 1, lane B). If Fru-P_2 was included in the cytoplasmic phosphorylation assays, phosphorylation of the 15 KD-protein was enhanced, while that of the 61 KD-protein was inhibited (Fig. 1, lane C). Inclusion of Fru-P_2 in membrane phosphorylation assays had little effect on the labeling pattern (not shown). In contrast to the results with Fru-P_2, F1P, 2PG, and 3PG inhibited phosphorylation of the 15-KD protein and stimulated that of the 61-KD protein in cytoplasmic fractions (Fig. 1, lanes D–F) as did dihydroxyacetone phosphate (Table I). Other glycolytic intermediates and inorganic phosphate inhibited phosphorylation of either or both proteins and all of these results are quantitated in Table I.

Identification of the 15-KD Protein

Using similar procedures, Deutscher and Saier [4] identified the phosphocarrier protein HPr of the PTS as the predominant phosphorylated polypeptide in *Streptococ-*

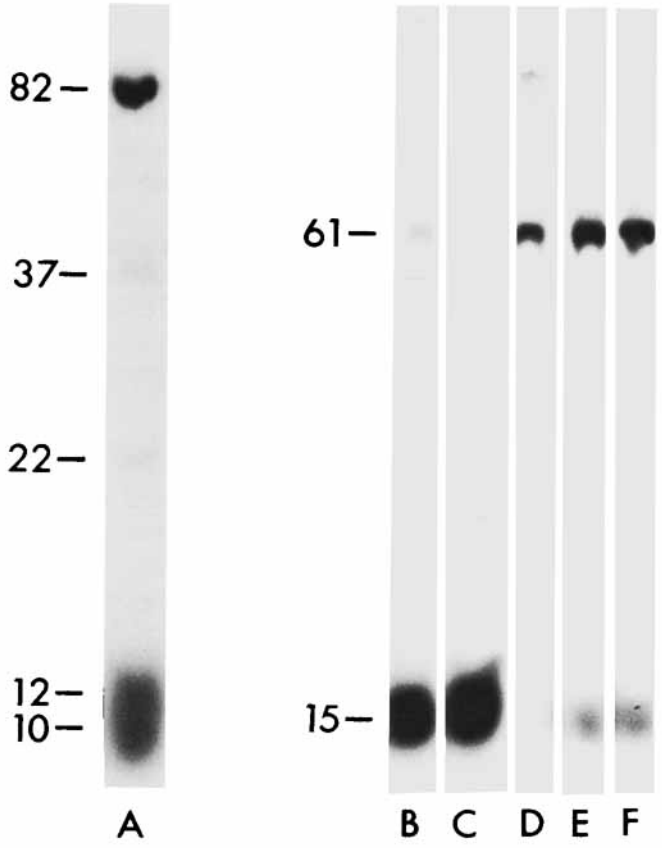

Fig. 1. ATP-dependent kinase activities in membrane and cytoplasmic fractions of *S mutans*. Each reaction mixture (35 μl) was prepared and electrophoresed as described in "Methods." The specific radioactivity of the γ-[^{32}P] ATP was 3.4 mCi/μmol, and each reaction mixture contained either 130 μg membrane protein (lane A) or 110 μg cytoplasmic protein (lanes B–F). Any other additions are as detailed below. Lanes: A, membrane fraction, no additions; B, cytoplasmic fraction, no additions; C, cytoplasmic fraction plus 4 mM Fru-P_2; D, cytoplasmic fraction plus 4 mM F1P; E, cytoplasmic fraction plus 4 mM 2PG; F, cytoplasmic fraction plus 4 mM 3PG.

cus pyogenes in the presence of certain glycolytic intermediates including Fru-P_2. Because one protein phosphorylated in cytoplasmic fractions of *S. mutans* had an apparent molecular weight similar to purified HPr from this organism (17 KD [ref. 14]), we measured kinase activity in the presence and absence of IgG prepared from antiserum against *S mutans* HPr. Table II shows that this antiserum was effective in inhibiting PEP-dependent phosphorylation of sucrose and glucose, both of which are phosphorylated and transported in *S mutans* by a PTS [18–20] that requires participation of HPr in the phosphotransfer reactions [14]. In Figure 2 (A and B) we show that anti-HPr IgG also inhibited ATP-dependent phosphorylation of the 15-KD protein in cytoplasm from *S mutans*, while the phosphorylation of a second protein (13 KD), which was occasionally also observed, was actually slightly enhanced under these conditions. Anti-HPr IgG had no effect on ATP-dependent phosphorylation in membranes nor did it inhibit phosphorylation of the 61-KD protein (not shown). Analysis of *S mutans* cytoplasmic proteins that interact with anti-HPr IgG by immunoblotting

TABLE I. Quantitation of Phosphorylation of the Cytoplasmic 15- and 61-KD Proteins in the Absence and Presence of Glycolytic Intermediates*

Addition (4 mM unless otherwise indicated)	Amount of label (%)[a]	
	15-KD protein	61-KD protein
None	100	100
Fru-P_2	162	12
FlP	15	382
2PG	11	708
3PG	9	811
Glucose 1-phosphate	95	< 10
Glucose 6-phosphate	100	< 10
Fructose 6-phosphate	85	< 10
Dihydroxyacetone phosphate	58	182
Glyceraldehyde 3-phosphate	114	384
2,3-diphosphoglycerate	20	129
Inorganic phosphate (5 mM)	10	82
Inorganic phosphate (50 mM)	2	< 10

*Determined from densitometric scans of autoradiograms.
[a]Relative units. The amount of phosphorylation of each protein in the absence of glycolytic intermediates was assigned a value of 100%; under these conditions the amount of label in the 15-KD protein was 46 times the amount in the 61-KD protein (cf. also Fig. 1). These results are averages of four independent experiments for the 15-KD protein and three independent experiments for the 61-KD protein.

TABLE II. Inhibition of PTS Activities by Anti-HPr Antiserum*

Antiserum	Sucrose-6-P formed (nmol/hr·mg)[a]	Glucose-6-P formed (nmol/hr·mg)[a]
Preimmune (780 μg)	9.5 (0)[b]	21.5 (0)
Anti-*S mutans* HPr (750 μg)	4.6 (52)	9.7 (55)

*PEP-dependent phosphorylation assays were performed using membrane (145 μg) and cytoplasmic (33 μg) fractions prepared as described [14].
[a]Specific activities are relative to the amount of *S mutans* cytoplasmic protein present.
[b]The Nos. in parentheses refer to the percent inhibition in the presence of the stated antisera.

revealed only one immunoreactive band that comigrated with the 15-KD protein (Fig. 2C). Thus, in *S mutans* HPr is one target of ATP-dependent protein kinase activity, as it is in several other gram-positive bacteria [8].

Identification of the Phosphorylated Amino Acids in the Cytoplasmic Fraction

Several lines of evidence argue that the phosphorylations we observed in the cytoplasmic fraction were truly ATP dependent and were not due to the conversion of ATP to PEP via pyruvate kinase and subsequent PEP-dependent phosphorylations of PTS proteins. First, phosphohistidines formed in the latter reaction would not be stable to the acidic conditions used to fix and stain the gels prior to autoradiography [21]. Second, we used dialyzed cytoplasm, which should not contain pyruvate, a substrate for pyruvate kinase in the direction of PEP synthesis, and included KF in the reaction mixture to block conversion of any added carbohydrate to pyruvate. Also, *S mutans* pyruvate kinase requires glucose 6-phosphate for its activity [22], and this compound had little effect on phosphorylation of HPr and inhibited phosphoryla-

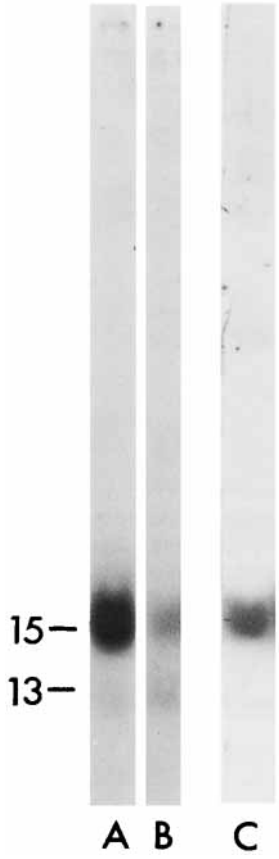

Fig. 2. Effect of anti-HPr IgG on protein phosphorylation in the cytoplasmic fraction of *S mutans*. Assays were conducted as in Figure 1 except that the specific radioactivity of the [^{32}P]ATP was 2.2 mCi/μmol, and the amount of cytoplasmic protein per assay was 55 μg. Lanes: A) cytoplasmic fraction, no additions; B) cytoplasmic fraction plus 30 μg anti-HPr IgG. Lane C is an immunoblot of unlabeled *S mutans* cytoplasm probed with anti-*S mutans* HPr IgG and ^{125}I-labeled staphylococcal protein A [17].

tion of the 61-KD protein (Table I). Finally, Figure 3 shows that the ATP-dependent phosphorylations of both of these proteins occur on serine residues. Acid hydrolysates of both phosphoproteins were separated by high-voltage electrophoresis, and autoradiography of the electropherograms identified the labeled amino acid as phosphoserine in both proteins.

Effects of PEP on ATP-Dependent Phosphorylations of *S mutans* Proteins

We have investigated the effect of prior formation of phospho-(histidyl)-HPr (resulting from PEP-dependent phosphorylation) on the formation of phospho-(seryl)-HPr. As shown in Table III, preincubation with PEP significantly decreased the amount of phospho-(seryl)-HPr produced by the ATP-dependent kinase when [^{32}P]ATP was subsequently added to the assay mixture. Addition of PEP simultaneously with, or after, addition of ATP did not significantly affect the amount of phospho-(seryl)-HPr formed (Table III). These results suggest not only that phospho-(histidyl)-HPr of *S mutans* is a poorer substrate of the kinase compared to free HPr but also that phosphorylation of HPr by the ATP-dependent kinase occurs faster under

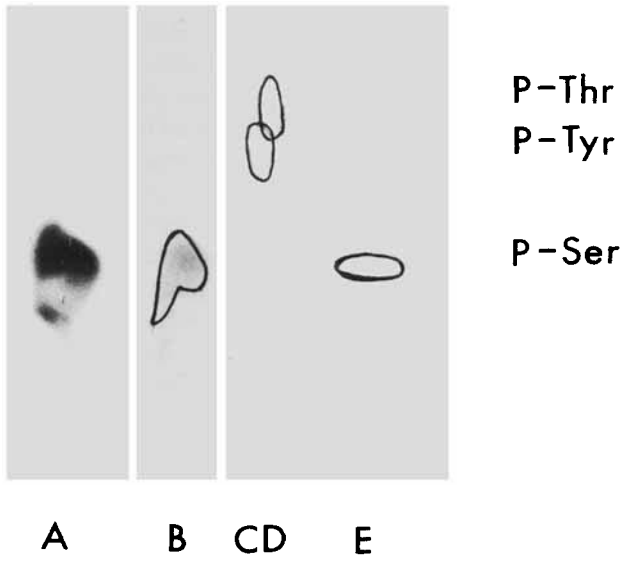

Fig. 3. Electrophoretic separation of hydrolyzed *S mutans* phospho-HPr and phospho-61-KD protein. Lanes A and B are autoradiograms of hydrolyzed phospho-61-KD protein and phospho-HPr, respectively. Lanes C, D, and E are ninhydrin-stained phosphotyrosine, phosphothreonine, and phosphoserine standards (1 μg each), respectively.

TABLE III. Quantitation of the Effects of PEP on the ATP-dependent Phosphorylation of HPr and the 61-KD Protein*

	Amount of label (%)[a]	
Incubation conditions	HPr	61-KD protein
Control ([^{32}P]ATP alone)	100	100
PEP Preincubation + [^{32}P]ATP	58	150
PEP + [^{32}P]ATP, added simultaneously	93	ND[b]
[^{32}P]ATP with PEP added 5 min later	106	ND

*Samples were prepared and incubated as described in the legend to Figure 1. Control conditions for HPr phosphorylation were those of Figure 1, lane B, and conditions for the 61-KD protein phosphorylation were those of Figure 1, lane D. PEP was present at 1 mM. Preincubation (second entry) was for 5 min at 37°C. For the last entry, the sample was incubated an additional 5 min at 37°C after PEP addition before electrophoresis and autoadiography. Data are from densitometric scans of autoradiograms and are averages of three experiments each.
[a]Relative units (see Table I.)
[b]Not determined.

these conditions than does PEP-dependent phosphorylation catalyzed by enzyme I of the PTS (cf. entries 2 and 3 in Table III). In contrast to the results obtained with HPr, preincubation with PEP did not diminish, but rather enhanced, the amount of the 61-KD phosphoprotein made in the ATP-dependent reaction (Table III).

Possible Identity of the 61-KD Protein

A cytoplasmic fraction of *S mutans* that was preincubated with [^{32}P]ATP in the presence of F1P was subjected to chromatography on Sephadex G-100, and the

phosphoproteins were detected in the eluate by electrophoresis and autoradiography. Figure 4 shows that the 61-KD phosphoprotein migrated at a position consistent with a native molecular weight of about 60,000 distinct from the elution position of PTS enzyme I activity. The same result was obtained when proteins were first fractionated on the column and then each fraction was tested in the kinase assay. Thus, the 61-KD protein is not enzyme I of the PTS, which in *S mutans* has a subunit molecular weight of about 60,000 but a native molecular weight near 120,000 (L. Eisenberg and G. Jacobson, unpublished). Another possibility is that the 61-KD protein could be the HPr kinase itself, which has been reported to have native and subunit molecular weights near 60,000 in *Streptococcus pyogenes* [8] and *Streptococcus faecalis* [11]. Consistent with this possibility, the peak fraction of the 61-KD protein from the Sephadex G-100 column was the most active one in catalyzing phosphorylation of HPr partially purified from the same column (not shown). Positive identification of

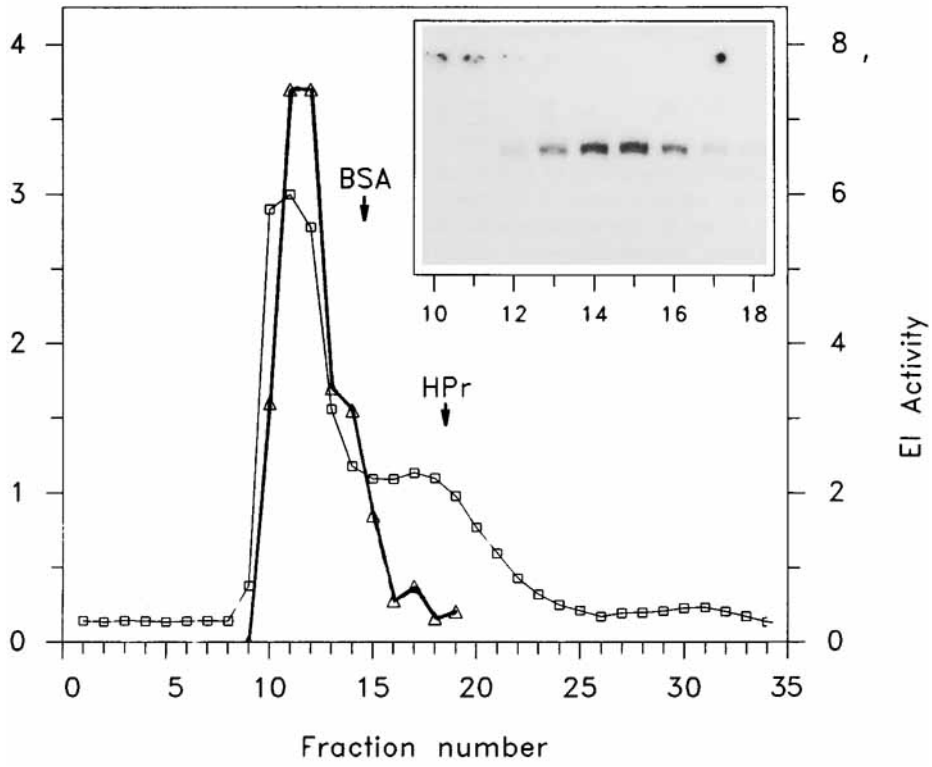

Fig. 4. Elution profile from a Sephadex G-100 column of phospho-61-KD protein. First 0.5 ml of *S mutans* cytoplasm was phosphorylated with [^{32}P]ATP in the presence of F1P as described in the legend to Figure 1. The sample was then chromatographed on Sephadex G-100 as described previously [14]. Each fraction (0.5 ml) was assayed for total protein (A_{280nm}; □—□) and PTS enzyme I (EI) activity. EI activity was measured as described [14] and is plotted as cpm × 10^{-3} of [^{14}C] pyruvate product formed (△—△). 25 μl of each fraction was electrophoresed and autoradiographed as described in "Methods." Inset: Shows that the phospho-61-KD protein migrated at the same position as bovine serum albumin (BSA) standard, with a peak at fraction 15. The elution position of HPr from the same column is also shown. In a second experiment, cytoplasm was first chromatographed, and then each fraction was assayed for kinase activity. The results were identical with those shown here, demonstrating that phosphorylation does not change the elution profile of the 61-KD protein (cf. also text).

the 61-KD protein, however, must await its purification to homogeneity (also see below).

DISCUSSION

The physiological significance of ATP-dependent phosphorylation of HPr [4,8,9, and this report] and of the 61-KD protein (this report) in gram-positive bacteria remains to be determined. However, Reizer, et al [8] had previously shown that ATP-dependent phosphorylation of *S pyogenes* HPr occurs on a seryl residue that is distinct from the histidyl residue that is phosphorylated by enzyme I of the PTS, and that phospho-(histidyl)-HPr is a poorer substrate than free HPr for ATP-dependent phosphorylation. In this report, we have shown that the same is true for *S mutans* HPr (Fig. 3 and Table III). Furthermore, as expected from the preceding observations, Deutscher et al [12] have shown that in *S pyogenes* phospho-(seryl)-HPr is resistant to PEP-dependent phosphorylation by enzyme I of the PTS. These results suggest that physiological conditions favoring the formation of phospho-(seryl)-HPr would have the overall consequence of inhibiting PTS-mediated uptake of carbohydrates in streptococci.

In *S mutans*, Fru-P_2 stimulated phosphorylation of HPr and inhibited phosphorylation of the 61-KD protein. Fru-P_2-stimulated phosphorylation of HPr has also been observed in other gram-positive bacteria [8,11]. In contrast, PEP, F1P, 2PG, and 3PG all enchanced phosphorylation of the 61-KD protein and inhibited that of HPr in *S mutans*. If the physiological consequence of ATP-dependent phosphorylation is to inhibit the uptake of at least some sugars, then our results, taken together with those of others, are consistent with the following regulatory model. Under conditions of glucose excess, *S mutans* and other streptococci have been shown to build up relatively high intracellular levels of Fru-P_2, while starved or glucose-limited cells have low levels of Fru-P_2 and high levels of 2PG, 3PG, PEP, and Pi [23,24]. Under the former condition, Fru-P_2 would be expected to stimulate ATP-dependent phosphorylation of HPr and thus to act as a feedback inhibitor of sugar uptake. On the other hand, starved or glucose-limited cells would contain HPr predominantly in the unphosphorylated (high activity) state as the combined consequence of low levels of Fru-P_2 and high levels of PEP, 2PG, 3PG, and Pi. This would ensure coordination of sugar uptake with sugar catabolism and preclude unnecessary uptake of sugars under conditions of excess availability.

Recently, Deutscher and Engelmann [11] have reported the purification of an HPr kinase from *S faecalis* that was stimulated by Fru-P_2 and inhibited by Pi, while Deutscher et al [25] have purified a phospho-(seryl)-HPr phosphatase from the same organism that was stimulated by Pi and inhibited by ATP. If *S mutans* contains a phosphatase that is regulated in a manner similar to that of *S faecalis*, then all these results would be consistent with the regulatory model presented above (see also [11,25] for a similar model in *S faecalis*).

A second way in which kinase-mediated phosphorylation of PTS proteins could regulate sugar catabolism in *S mutans* could be by means of inducer exclusion and/or inducer expulsion mechanisms [8,12]. Although PTS-mediated inducer expulsion has been observed in *S pyogenes* and *S lactis* [26–28], it has yet to be observed in *S mutans*. However, inducer exclusion models for regulation of hexitol [29] and lactose [30] uptake by the *S mutans* glucose PTS have been presented, and it is possible that

the ATP-dependent phosphorylation of HPr and/or the 61-KD protein described in this report could have some role in this process.

Recently, Waygood et al [9] have reported ATP-dependent phosphorylations of HPr in another oral streptococcus, *S salivarius*. Their results suggest that HPr in this organism can be phosphorylated by ATP on at least two different residues: the N-3 position of a histidine (acid labile), and on another residue yielding an acid-stable phosphoamino acid. Since all of our gels were acid-fixed prior to autoradiography, 3-phosphohistidine would not have been detected in our experiments. Whether *S mutans* HPr, phosphorylated with ATP, also contains acid-labile phosphate remains to be determined, as does the regulatory significance of this type of phosphorylation.

The role of the cytoplasmic 61-KD phosphoprotein in *S mutans* also remains a matter of speculation. Our results would seem to rule out that this protein is enzyme I of the PTS, and, they suggest, but do not prove, that this protein could be the HPr kinase itself. As mentioned earlier, several compounds that inhibit HPr phosphorylation concomitantly enhance that of the 61-KD protein, while Fru-P_2 has the reverse effect. This suggests a relationship between the kinase-mediated phosphorylation of these two proteins. If the 61-KD phosphoprotein is an HPr kinase, then our results are consistent with the possibility that (self ?)-phosphorylation of the kinase may inhibit its ability to phosphorylate HPr. In this model, effectors of kinase activity would then regulate phosphorylation of the kinase, which in turn would affect the phosphorylation state and activity of HPr. Purification of the HPr kinase will be necessary to prove these hypotheses as well as to determine at what level the effectors of kinase activity act. This will be important since our results with unfractionated extracts could be explained either by direct action of glycolytic intermediates on the kinase(s), or, for example, by regulation at another level, possibly the HPr phosphatase.

Finally, it will be interesting to identify the other phosphorylated polypeptides we have observed and to measure patterns of phosphorylation at different stages of growth or under varying growth conditions as has been done for *E coli* [7]. Other compounds, individually or in combination, will also need to be tested as effectors of ATP-dependent phosphorylation. Moreover, we expect that more sensitive techniques such as two-dimensional gel electrophoresis [31] could reveal other phosphorylated polypeptides that are present in small amounts but that could have important regulatory roles in *S mutans*. Undoubtedly, more roles of protein kinases in the regulation of bacterial metabolism will be discovered in the near future.

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