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Properties of a *Streptococcus salivarius* Spontaneous Mutant in Which the Methionine at Position 48 in the Protein HPr Has Been Replaced by a Valine

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HPr is a protein of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) that participates in the concomitant transport and phosphorylation of sugars in bacteria. In gram-positive bacteria, HPr is also reversibly phosphorylated at a seryl residue at position 46 (Ser-46) by a metabolite-activated ATP-dependent kinase and a P_i-dependent HPr(Ser-P) phosphatase. We report in this article the isolation of a spontaneous mutant (mutant A66) from a streptococcus (*Streptococcus salivarius*) in which the methionine at position 48 (Met-48) in the protein HPr has been replaced by a valine (Val). The mutation inhibited the phosphorylation of HPr on Ser-46 by the ATP-dependent kinase but did not prevent phosphorylation of HPr by enzyme I or the phosphorylation of enzyme II complexes by HPr(His-P). The results, however, suggested that replacement of Met-48 by Val decreased the affinity of enzyme I for HPr or the affinity of enzyme II proteins for HPr(His-P) or both. Characterization of mutant A66 demonstrated that it has pleiotropic properties, including the lack of III_L^{Man}, a specific protein of the mannose PTS; decreased levels of HPr; derepression of some cytoplasmic proteins; reduced growth on PTS as well as on non-PTS sugars; and aberrant growth in medium containing a mixture of sugars.

The uptake of several sugars by oral streptococci is catalyzed by the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) (9). Utilizing PEP as one substrate, the system catalyzes the sequential phosphorylation of the general energy-coupling, non-sugar-specific proteins enzyme I (EI) and HPr, required for the uptake of all PTS sugars. In many cases, phosphohistidine-HPr [HPr(His-P)], generated from phospho-EI, transfers the phosphoryl group directly to a carbohydrate-specific, membrane-bound enzyme IICBA (IICBA), which in turn phosphorylates the incoming sugar. HPr(His-P) may also transfer the phosphoryl group to a third cytoplasmic or membrane-bound protein called IIA (formerly called enzyme III) prior to interaction with the membrane component IICB, which assumes the permease function (13, 15, 21; see reference 23 for nomenclature rules and definitions).

In gram-positive bacteria, including oral streptococci (14, 25), HPr is reversibly phosphorylated at a seryl residue at position 46 (Ser-46) by a metabolite-activated ATP-dependent kinase and a P_i-dependent HPr(Ser-P) phosphatase (3, 16, 18). It has been proposed that the phosphorylation of HPr on Ser-46 serves to rapidly modulate the activity of the PTS as a function of the energetic status of the cell (3, 16, 18). However, studies conducted with site-directed mutants of *Bacillus subtilis* (20, 22), as well as the discovery of HPr(Ser-P) and of the HPr(Ser) kinase in heterofermentative lactobacilli devoid of PTS activity (19), have suggested that the regulatory functions of HPr(Ser-P) are not restricted (and perhaps not related) to the modulation of PTS activity. By crystallographic studies of HPr from *B. subtilis*, Herzberg et al. (11) have shown that Ser-46 and His-15, the histidine residue phosphorylated by EI, are located on either side of a hydrophobic patch formed by Ile-47, Met-48, and Met-51, three amino acids also found in the HPr from other gram-positive bacteria (17), including *Streptococcus salivarius* (5). Herzberg et al. (11) have proposed that this hydrophobic pocket may be the region of interaction of HPr with EI and with the IIA domains of the PTS permeases as well as with the HPr kinase. We report in this article the isolation and characterization of a spontaneous *ptsH* mutant of *S. salivarius* in which Met-48 in the protein HPr has been replaced by Val.

Mutant A66 was isolated as a spontaneous mutant resistant to the toxic effect of 2-deoxyglucose (2DG), a nonmetabolizable mannose analog, by plating 100 μl of a mid-log-phase culture (optical density at 660 nm, 0.45) of *S. salivarius* ATCC 25975 grown in 0.2% glucose onto a Trypticase-yeast extract-agar medium containing (per liter) 17 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 3 g of yeast extract (Difco Laboratories, Detroit, Mich.), 5 g of NaCl, 2.5 g of disodium phosphate, 3.6 g of lactose, and 0.082 g of 2DG. Strain A66 was unable to take up 2DG and was devoid of in vitro mannose PTS activity (not shown). Determination of the intracellular concentrations of the energy-coupling proteins HPr and EI by rocket immunoelectrophoresis (7) also indicated that the mutant contained almost threefold less HPr than the parental strain, whereas the level of EI was slightly higher (not shown). To determine whether this reduced level of HPr was caused by a mutation in the *ptsH* gene, encoding HPr, we amplified the *ptsH* gene from mutant A66 by PCR and sequenced it. Two oligonucleotides, a 17-mer (5′-GATGCT GCGATTCCTTT-3′) and a 22-mer (5′-ACCAGTAAAAT GAGAGACAAGC-3′), were synthesized with an Applied Biosystems model 392 DNA synthesizer (Applied Biosystems Inc., Mississauga, Ontario, Canada). These primers annealed downstream and upstream, respectively, from the *ptsH* gene, allowing the amplification of a DNA fragment encompassing the entire *ptsH* gene, the 5′ end of *ptsI*, and the transcriptional

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regulatory elements (5, 6). PCR was performed with a DNA Thermal Cycler 480 (Perkin Elmer) and genomic DNA purified as described previously (6). The amplicon was purified with a Magic PCR Prep Kit (Promega). The DNA sequence was determined by the dideoxy chain termination method (24) with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corporation), [α^{35}S]dATP, and the above-described oligonucleotides as primers. The Genetics Computer Group software package was used to analyze the DNA and amino acid sequences (4). By comparison with the wild-type (5), it was found that the *ptsH* gene from mutant A66 had an A-to-G base substitution at nucleotide position 142, causing a missense mutation which resulted in a Met-to-Val conversion at amino acid 48 of the HPr protein.

The Met-48 → Val mutation in A66 HPr occurred in the vicinity of Ser-46, the phosphorylation target of the ATP-dependent HPr kinase in gram-positive bacteria (18). Met-48 is conserved in the known sequences of HPrs from gram-positive bacteria (17), and it has been proposed that replacement of this residue may impede the phosphorylation of HPr on Ser-46 (11). To test this hypothesis, we measured the in vitro ATP-dependent phosphorylation of HPr by using membrane fragments of the mutant and the wild-type strains as sources of kinase and purified HPr as well as membrane-free cellular extracts of the wild-type strain and mutant A66 as sources of HPr. Membrane fragments were prepared in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.0) as described previously (1). Samples containing 50 μg of protein were incubated at 37°C for 10 min with 2.5 to 5 μg of either purified HPr or membrane-free cellular extract containing 20 μg of protein, 0.1 mM [γ-^{32}P]ATP (1.0 μCi/nmol), 5 mM fructose-1,6-diphosphate (FDP), 5 mM $MgCl_2$, and 12.5 mM NaF in 10 mM HEPES buffer (pH 7.0), in a total volume of 30 μl. After incubation, the samples were mixed with 30 μl of a solution containing 125 mM Tris-HCl (pH 8.0), 2% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue. Samples were boiled for 3 min (HPr-Ser-P is resistant to heat) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (12), with resolving gels containing 10% acrylamide. The gels were then analyzed by autoradiography. The results (Fig. 1) indicated that membranes of the wild-type and mutant strains could catalyze the ATP-dependent phosphorylation of purified wild-type HPr and of HPr present in the membrane-free cellular extract of the wild-type strain (Fig. 1, lanes 1 and 4 to 8), indicating that the HPr(Ser) kinase of the mutant strain was active. Membrane fragments of both strains were, however, unable to phosphorylate either the HPr present in a membrane-free cellular extract of mutant A66 (Fig. 1, lanes 2 and 3) or purified A66 HPr (not shown), indicating that the Met-48 to Val mutation impeded the phosphorylation of HPr on Ser-46.

In order to determine whether the conversion of Met-48 to Val altered the interaction of HPr with EI or the interaction of HPr(His-P) with EII proteins, we used an in vitro PTS assay (10) to measure the rate of phosphorylation of fructose under conditions of HPr limitation. Fructose was chosen because its PEP-dependent phosphorylation does not require the presence of a specific soluble IIA protein (2). We found that replacing the wild-type HPr with the A66 HPr in the reaction mixture resulted in a twofold decrease in the rate of phosphorylation of fructose (Fig. 2).

Our results suggested that Met-48 is essential for the interaction of HPr with the HPr kinase. It has been proposed previously that the HPr kinase recognizes the three-dimensional structure of HPr rather than a restricted amino-acyl

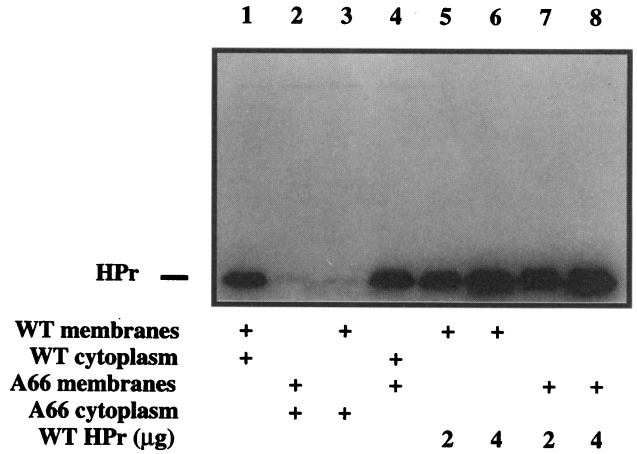

FIG. 1. In vitro phosphorylation of HPr by the ATP-dependent HPr kinase. After growing cells of the wild-type (WT) strain and mutant A66 in the presence of 0.5% glucose, cytoplasmic proteins (source of HPr) were separated from membranes (source of HPr kinase) and incubated in the presence of [γ-^{32}P]ATP as described in the text. Electrophoresis and autoradiography were carried out as described by Waygood et al. (25). Lanes 5 to 8 contained purified wild-type HPr. The components of the reaction mixes are indicated below the autoradiogram.

sequence within the protein (22). We therefore propose that the conversion of Met-48 to Val results in a serious perturbation in the structure of the HPr protein. Moreover, since Met and Val cannot substitute for each other, even though both amino acids have nonpolar side chains, it appears that the identity of the side chain at position 48 is a critical determinant of the protein structure. This modification of the tertiary structure of the protein did not prevent the phosphorylation of HPr by EI or the phosphorylation of EII complexes by HPr(His-P). Our results, however, indicated that replacement of Met-48 by Val decreased the affinity of EI for HPr or the affinity of II proteins for HPr(His-P) or both.

Characterization of mutant A66 demonstrated that it possessed pleiotropic properties. We have observed that the growth of this mutant was reduced on PTS (glucose, fructose, and mannose) as well as on non-PTS (lactose, galactose, and melibiose) sugars (Table 1) and that the control of sugar utilization by glucose and fructose was impaired (not shown). Moreover, rocket immunoelectrophoresis and two-dimensional gel electrophoresis analyses indicated that the HPr level was decreased threefold and that five glucose-repressible genes encoding cytoplasmic proteins were derepressed in glucose-grown cells of mutant A66 (not shown). Finally, we have shown by Western blot (immunoblot) experiments that the mutant lacked III_L^{Man}, a specific component of the mannose PTS (Fig. 3). This last observation explained the inability of mutant A66 to take up 2DG and to grow on mannose and raised the question of whether the absence of III_L^{Man} was a consequence of the mutation in the *ptsH* gene or resulted from a second distinct mutation.

It was reasoned that if the synthesis of III_L^{Man} was actually a consequence of the mutation which prevented the phosphorylation of HPr on Ser-46, isolation of revertants of strain A66 that have recovered the ability to grow on mannose should allow the isolation of clones producing normal levels of III_L^{Man} and of HPr(Ser-P). We have not, however, been able to isolate revertants able to grow on mannose, perhaps because only one nucleotide substitution (G to A) can convert the mutant allele

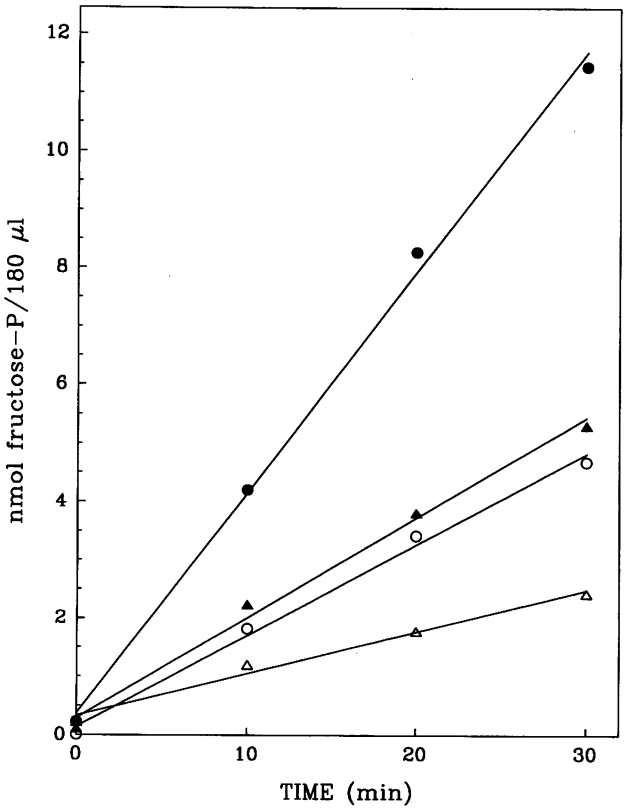

FIG. 2. Rates of PTS-catalyzed phosphorylation of fructose. The reaction mixture (800 µl) was incubated at 37°C and contained 50 mM sodium phosphate (pH 7.0), 4 mM $MgCl_2$, 2 mM PEP, 5 mM 2-mercaptoethanol, 10 mM NaF, 1 mM [U-^{14}C]fructose (0.1 µCi/µmol), excess EI (5.6 µg), membranes (66.7 µg of protein) from *S. salivarius* grown on 0.5% fructose, and 20 µg (●) or 40 µg (▲) of wild-type HPr or 20 µg (○) or 40 µg (△) of A66 HPr. At the indicated times, 180-µl samples were withdrawn, and the phosphorylated product (fructose-P) was separated by precipitation with 10 volumes of $BaBr_2$ in 90% ethanol (1).

to the wild-type allele, as there is only one codon for Met. Thus, the likelihood of isolating a true spontaneous or chemically induced revertant would be very low. We cannot, however, exclude the possibility that A66 is a double spontaneous mutant, with a mutation in the *ptsH* gene and another mutation in a second gene preventing the synthesis of III_L^{Man}. Recent data have indeed suggested that bacteria possess a mechanism that increases the rate of multiple spontaneous mutations when these are required for survival (8). Consequently, the pleiotropic properties of mutant A66 could have resulted from the inability of the cell to produce HPr(Ser-P) or could be caused by a second mutation preventing the synthesis of III_L^{Man}.

TABLE 1. Generation times of wild-type strain and mutant A66

Sugar (0.2%)	Doubling time (min) at 37°C	
	Wild type	A66
PTS		
Glucose	30	95
Mannose	40	>300
Fructose	30	60
Non-PTS		
Lactose	30	40
Galactose	33	60
Melibiose	30	45

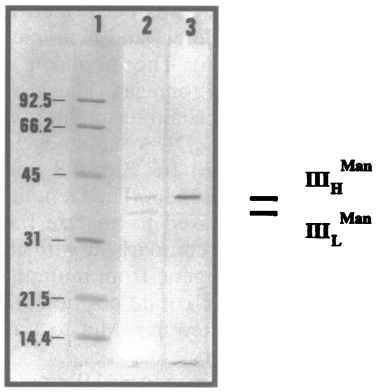

FIG. 3. Western analysis with anti-III_H^{Man} antibody. Samples containing 35 µg of protein were electrophoresed in 10% acrylamide gels by the method of Laemmli (12) and electrophoretically transferred to a nitrocellulose sheet as described previously (1). Sizes are shown in kilodaltons. Lane 1, prestained molecular size standards from Bio-Rad; lane 2, membrane-free cellular extract from *S. salivarius* ATCC 25975; lane 3, membrane-free cellular extract from mutant A66. The sample in lane 3 was incubated for a longer period in the presence of the anti-rabbit immunoglobulin conjugate to test for very small amounts of III_H^{Man} in mutant A66.

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REFERENCES

1. **Bourassa, S., L. Gauthier, R. Giguère, and C. Vadeboncoeur.** 1990. A III^{Man} protein is involved in the transport of glucose, mannose, and fructose by oral streptococci. Oral Microbiol. Immunol. **5**:288–297.
2. **Bourassa, S., and C. Vadeboncoeur.** 1992. Expression of an inducible enzyme II fructose and activation of a cryptic enzyme II glucose in glucose-grown cells of spontaneous mutants of *Streptococcus salivarius* lacking the low-molecular-mass form of III^{Man}, a component of the phosphoenolpyruvate:mannose phosphotransferase system. J. Gen. Microbiol. **138**:769–777.
3. **Deutscher, J., G. Sossna, and G. Gonzy-Treboul.** 1989. Regulatory functions of the phosphocarrier protein HPr of the phosphoenolpyruvate-dependent phosphotransferase system in Gram-positive bacteria. FEMS Microbiol. Lett. **63**:167–174.
4. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
5. **Gagnon, G., C. Vadeboncoeur, and M. Frenette.** 1993. Phosphotransferase system of *Streptococcus salivarius*: characterization of the *ptsH* gene and its product. Gene **136**:27–34.
6. **Gagnon, G., C. Vadeboncoeur, R. C. Levesque, and M. Frenette.** 1992. Cloning, sequencing and expression in *Escherichia coli* of the *ptsI* gene encoding the enzyme I of the phosphoenolpyruvate:sugar phosphotransferase transport system from *Streptococcus salivarius*. Gene **121**:71–78.
7. **Gauthier, L., S. Bourassa, D. Brochu, and C. Vadeboncoeur.** 1990. Control of sugar utilization in oral streptococci. Properties of

phenotypically distinct 2-deoxyglucose-resistant mutants of *Streptococcus salivarius*. Oral Microbiol. Immunol. **5**:352–359.
8. **Hall, B. G.** 1991. Adaptive evolution that requires multiple spontaneous mutations: mutations involving base substitutions. Proc. Natl. Acad. Sci. USA **88**:5882–5886.
9. **Hamilton, I. R.** 1987. Effect of changing environment on sugar transport and metabolism by oral bacteria, p. 94–133. *In* J. Reizer and A. Peterkofsky (ed.), Sugar transport and metabolism by Gram-positive bacteria. Ellis Horwood, Chichester, England.
10. **Hamilton, I. R., L. Gauthier, B. Desjardins, and C. Vadeboncoeur.** 1989. Concentration-dependent repression of the soluble and membrane components of the *Streptococcus mutans* phosphoenolpyruvate:sugar phosphotransferase system by glucose. J. Bacteriol. **171**:2942–2948.
11. **Herzberg, O., P. Reddy, S. Sutrina, M. H. Saier, Jr., J. Reizer, and G. Kapadia.** 1992. Structure of the histidine-containing phosphocarrier protein HPr from *Bacillus subtilis* at 2.0-Å resolution. Proc. Natl. Acad. Sci. USA **89**:2499–2503.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Meadow, N. D., D. K. Fox, and S. Roseman.** 1990. The bacterial phosphoenolpyruvate:glycose phosphotransferase system. Annu. Rev. Biochem. **59**:497–542.
14. **Mimura, C. S., F. Poy, and G. R. Jacobson.** 1987. ATP-dependent protein kinase activities in the oral pathogen *Streptococcus mutans*. J. Cell. Biochem. **33**:161–171.
15. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57**:543–594.
16. **Reizer, J., J. Deutscher, and M. H. Saier, Jr.** 1989. Metabolite-sensitive, ATP-dependent, protein kinase-catalyzed phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system in Gram-positive bacteria. Biochimie **71**:989–996.
17. **Reizer, J., C. Hoischen, A. Reizer, T. N. Pham, and M. H. Saier, Jr.** 1993. Sequence analyses and evolutionary relationships among the energy-coupling proteins enzyme I and HPr of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Protein Sci. **2**:506–521.
18. **Reizer, J., and A. Peterkofsky.** 1987. Regulatory mechanisms for sugar transport in Gram-positive bacteria, p. 333–364. *In* J. Reizer and A. Peterkofsky (ed.), Sugar transport and metabolism by Gram-positive bacteria. Ellis Horwood, Chichester, England.
19. **Reizer, J., A. Peterkofsky, and A. H. Romano.** 1988. Evidence for the presence of heat-stable protein (HPr) and ATP-dependent HPr kinase in heterofermentative lactobacilli lacking phosphoenolpyruvate:glucose phosphotransferase activity. Proc. Natl. Acad. Sci. USA **85**:2041–2045.
20. **Reizer, J., A. H. Romano, and J. Deutscher.** 1993. The role of phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, in the regulation of carbon metabolism in Gram-positive bacteria. J. Cell. Biochem. **51**:19–24.
21. **Reizer, J., M. H. Saier, Jr., J. Deutscher, F. Grenier, J. Thompson, and W. Hengstenberg.** 1988. The phosphoenolpyruvate:sugar phosphotransferase system in Gram-positive bacteria:properties, mechanism, and regulation. Crit. Rev. Microbiol. **15**:297–338.
22. **Reizer, J., S. L. Sutrina, M. H. Saier, Jr., G. C. Stewart, A. Peterkofsky, and P. Reddy.** 1989. Mechanistic and physiological consequences of HPr(ser) phosphorylation on the activities of the phosphoenolpyruvate:sugar phosphotransferase system in Gram-positive bacteria: studies with site-specific mutants of HPr. EMBO J. **8**:2111–2120.
23. **Saier, M. H., Jr., and J. Reizer.** 1992. Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. J. Bacteriol. **174**:1433–1438.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
25. **Waygood, E. B., R. L. Mattoo, E. Erickson, and C. Vadeboncoeur.** 1986. Phosphoproteins and the phosphoenolpyruvate:sugar phosphotransferase system of *Streptococcus salivarius*. Detection of two different ATP-dependent phosphorylations of the phosphocarrier HPr. Can. J. Microbiol. **32**:310–318.