Surface location of HPr, a phosphocarrier of the phosphoenolpyruvate:sugar phosphotransferase system in *Streptococcus suis*

J. D. Dubreuil,¹ M. Jacques,¹ D. Brochu,² M. Frenette²
and C. Vadeboncoeur²

Author for correspondence: C. Vadeboncoeur. Tel: +1 418 656 2319. Fax: +1 418 656 2861.
e-mail: Christian.Vadeboncoeur@greb.ulaval.ca

¹ Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Université de Montréal, PO Box 5000, Saint Hyacinthe, Québec, Canada J2S 7C6

² Groupe de Recherche en Ecologie Buccale (GREB), Department of Biochemistry (Sciences) and Faculty of Dentistry, Université Laval, Québec, Canada G1K 7P4

HPr is a low-molecular-mass phosphocarrier protein of the bacterial phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) found in the cytoplasm or associated with the inner surface of the cytoplasmic membrane. Treatment of *Streptococcus suis* cells with a Sorvall Omnimixer, a technique used to extract cell surface components, resulted in the extraction of a major protein with a molecular mass of 9 kDa. Several lines of evidence suggested that this protein was HPr: (i) the *S. suis* protein showed homology over the first 35 N-terminal amino acid residues with the HPrs of *Streptococcus salivarius* and *Streptococcus mutans*, including the signature sequence for the site of PEP-dependent phosphorylation; (ii) it cross-reacted with the *S. salivarius* anti-HPr antibody preparation; (iii) it could be phosphorylated by enzyme I at the expense of PEP, and by a membrane-associated kinase at the expense of ATP; and (iv) it possessed phosphocarrier activity when used as a source of HPr in an *in vitro* PTS assay. The data suggested that a portion of the cellular HPr is associated with the external cell surface in *S. suis*, a result that was confirmed by immunogold electron microscopy. The cellular HPr of *S. suis* consisted of two forms that could be distinguished by the presence or the absence of the N-terminal methionine. Amino acid sequence analysis indicated that the cell-surface-associated HPr of *S. suis* lacked the N-terminal methionine residue.

Keywords: HPr, phosphotransferase system, cell surface proteins, *Streptococcus suis*, methionine aminopeptidase

INTRODUCTION

Streptococci use mainly sugars as energy sources and, in many instances, transport them by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) (Hamilton, 1987; Thompson, 1987). This transport system concomitantly catalyses the transport and the phosphorylation of several mono- and disaccharides at the expense of phosphoenolpyruvate (PEP) (Meadow *et al.*, 1990; Postma *et al.*, 1993). The PTS is composed of several soluble and membrane-associated proteins, and

transfers the phosphate group from PEP to the incoming sugar in an orderly sequence (Meadow *et al.*, 1990; Postma *et al.*, 1993). The first step of this energy-conserving cascade is catalysed by Enzyme I (EI), which phosphorylates a small protein, designated HPr, on a histidine residue at position 15 at the expense of PEP. Thereafter, HPr(His ~ P) transfers its phosphate group to a family of multi-domain protein complexes designated Enzyme IIs (EII) that catalyse the transport and phosphorylation of sugars (Saier & Reizer, 1992).

Several results suggest that HPr possesses other physiological functions besides its role as a phosphocarrier in sugar transport. The HPrs of several Gram-positive bacteria and mycoplasmas, including species that do not possess an active PTS, can be reversibly phosphorylated on a serine residue at position 46 by an ATP-dependent

Abbreviations: EI, enzyme I; EII or II, enzyme II; PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate:sugar phosphotransferase system.

The N-terminal amino acid sequence of *S. suis* HPr will appear in the PIR database under the accession number A57222.

protein kinase and a P_i -dependent HPr(Ser-P) phosphatase (Deutscher *et al.*, 1989; Postma *et al.*, 1993; Reizer *et al.*, 1989, 1993b). The various regulatory roles that have been assigned to HPr(Ser-P) were recently reviewed (Vadeboncoeur, 1995).

In streptococci and lactococci, HPrs are subjected to further chemical modification consisting of a partial cleavage of the N-terminal methionine (Met), giving rise to two forms of HPr that are designated HPr-1 (without Met) and HPr-2 (with Met) (Robitaille *et al.*, 1991; Vadeboncoeur *et al.*, 1991b). These two forms of HPr can be phosphorylated at the expense of PEP by the EI of the PTS and by the HPr-specific ATP-dependent kinase. The physiological relevance of the partial removal of the N-terminal Met in HPr is unclear, but the HPr-1/HPr-2 ratio varies in *Streptococcus salivarius* according to growth conditions (Robitaille *et al.*, 1991; Vadeboncoeur *et al.*, 1993). Circumstantial evidence is, however, accumulating that streptococcal HPrs lacking the N-terminal Met may be surface-located: (1) using an extraction method that did not disrupt cells, Jenkinson (1989) isolated the *Streptococcus sanguis* HPr and showed by sequence analysis that the purified protein lacked the N-terminal Met; (2) a low-molecular-mass protein lacking the N-terminal Met, and possessing 100% identity with the 19 N-terminal amino acids of the *S. salivarius* HPr, was purified from a *Streptococcus pyogenes* culture supernatant (Gerlach *et al.*, 1992); (3) Sutcliffe *et al.* (1993) identified the *Streptococcus mutans* surface antigen D as an HPr, and demonstrated that it lacked the N-terminal Met, whereas its cytosolic counterpart was a mixture of HPr-1 and HPr-2.

Streptococcus suis is associated with different swine (Higgins *et al.*, 1990), ruminant (Hommeze *et al.*, 1986) and human (Arends & Zanen, 1988; Trottier *et al.*, 1991) pathologies. Results presented here demonstrate that this bacterial species possesses the two forms of HPr, designated HPr-1 and HPr-2, and provide for the first time convincing direct evidence that a portion of the cellular HPr-1 is located at the cell surface.

METHODS

Bacterial strains and growth conditions. *S. suis* strain 170B, isolated from swine lungs and belonging to serotype 19, was obtained from Dr R. Higgins (Clinical Diagnostic Laboratory, Faculty of Veterinary Medicine, St Hyacinthe, Québec, Canada). *S. salivarius* ATCC 25975 was kindly provided by Dr I. R. Hamilton (University of Manitoba, Canada). *S. suis* was grown in Todd-Hewitt broth for 18 h at 37 °C with agitation (200 r.p.m.). *S. salivarius* was grown at 37 °C without agitation in TYE medium containing 17 g Trypticase (BBL Microbiology Systems), 3 g yeast extract (Difco), 5 g NaCl, 2.5 g disodium phosphate and 50 g glucose or fructose per litre.

Extraction of surface components. A 350 ml culture of *S. suis* was grown as described above, harvested by centrifugation at 10000 g for 30 min and resuspended in 0.01 M sodium phosphate (pH 7.2) containing 1 mM PMSF. Cells received two 5 min treatments on ice in a model 17105 Sorval Omni-mixer (Dupont Instruments) at 15000 r.p.m., a technique used to isolate bacterial surface appendages (Heckels & Virji, 1988). After centrifugation of the blended preparation (10000 g for

30 min), urea was added to the supernatant to a final concentration of 6 M. The solution was kept at 4 °C for 60 min and then centrifuged at 25000 g for 90 min. The supernatant was filtered through a 0.22 µm Millipore membrane, dialysed against distilled water and concentrated using an Amicon ultrafiltration unit fitted with a YM 30 membrane.

Amino acid sequence analysis. Automated Edman degradation was performed in a gas-phase sequencer (model 470A) equipped with an on-line phenylthiohydantoin analyser (model 120A) from Applied Biosystems using the general protocol of Hewick *et al.* (1980). The sample was applied to a trifluoroacetic-acid-treated cartridge filter coated with 1.5 mg polybrene and 0.1 mg NaCl (Biobrene Plus, ABI). A standard program (03RPTH, ABI) was used for sequencing. The phenylthiohydantoin amino acid derivatives were identified by comparison with standards (PTH Analyser standards, ABI) analysed on-line prior to sequence analysis. Sequence comparison was carried out using the GCG sequence analysis software package (Devereux *et al.*, 1984).

Electrophoretic procedures. PAGE in the presence of SDS and urea was carried out as described by Swank & Munkres (1971), and proteins were stained with Coomassie blue. The separation of HPr-1 and HPr-2 by non-denaturing electrophoresis was carried out as described previously (Robitaille *et al.*, 1991). For Western blotting, proteins were transferred electrophoretically at 4 V cm⁻¹ overnight to a nitrocellulose membrane (0.45 µm pore size) according to Towbin *et al.* (1979). Following transfer, the membrane was treated for 60 min with 2% casein in 10 mM Tris/HCl (pH 8.0) containing 150 mM NaCl to block unoccupied sites, and then incubated with rabbit anti-*S. salivarius* HPr antiserum for 90 min at room temperature. The membrane was then washed three times with 10 mM Tris/HCl (pH 8.0) containing 150 mM NaCl, incubated with peroxidase-conjugated anti-rabbit immunoglobulin G for 60 min, and developed with 10 ml 0.3% 4-chloro-1-naphthol (Sigma) in cold methanol mixed with 50 ml 0.06% H₂O₂ in Tris-saline (10 mM Tris/HCl, 0.9% NaCl, pH 7.4) (Hawkes, 1982). Crossed immunoelectrophoresis was conducted as described by Vadeboncoeur *et al.* (1991a). Antiserum against HPr was obtained from female New Zealand White rabbits after immunization by multisite subcutaneous injection (Gauthier *et al.*, 1984). Approximately 200 µg protein was injected for both the primary and booster injections.

PTS assay. *S. salivarius* 25975 was grown in the presence of 0.5% fructose and harvested at mid-exponential phase. Membrane fragments containing the PTS EII complexes were prepared as previously described (Hamilton *et al.*, 1989). The PTS-dependent phosphorylation of fructose was carried out in a reaction medium (600 µl) containing 50 mM sodium phosphate (pH 7.0), 4 mM MgCl₂, 2 mM PEP, 5 mM 2-mercaptoethanol, 10 mM NaF, 10 mM [¹⁴C]fructose [0.1 mCi mmol⁻¹ (37.5 kBq mmol⁻¹)], 1.5 µg purified *S. sanguis* EI, 16.5 µg purified HPr from *S. salivarius* or 15 µg of the 9 kDa protein extracted from *S. suis* and 20 µg *S. salivarius* membrane proteins as a source of EII complexes. After a pre-incubation of 10 min at 37 °C, the labelled sugar was added, and the reaction was allowed to continue for 1 h at 37 °C. The reaction was then stopped with 6 ml 0.03 M BaBr₂ in 90% (v/v) ethanol, and the phosphorylated product was allowed to precipitate for 20 min at 4 °C. It was then filtered through a 0.45 µm Millipore membrane and rinsed twice with 80% (v/v) cold ethanol (2 × 1.0 ml). The filter was placed in a vial containing 5.0 ml Scintiverse E (Fisher Scientific) and the radioactivity was counted using a Beckman LS 7500 liquid scintillation system. The activities were corrected with respect to a control consisting of the same incubation medium described above but without HPr.

Electron microscopy. Single drops of bacterial suspension were placed on Formvar-coated grids. After 1 min, the grids were placed on drops of 1% (w/v) egg albumin for 5 min and then in a suitable dilution of rabbit anti-*S. salivarius* serum for 30 min. They were then washed in distilled water and placed on drops of colloidal gold particles (10 nm) conjugated to goat anti-rabbit immunoglobulin G (Sigma) for 30 min. After a final wash in distilled water, they were stained with 0.2% (w/v) phosphotungstate (pH 7.0), and examined with a Philips 201 electron microscope at an accelerating voltage of 60 kV. Bacterial cells were also fixed for 2 h at room temperature in cacodylate buffer (0.1 M, pH 7.2) containing 1% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde, then dehydrated in ethanol and embedded in Lowicryl K4M at -20°C . Polymerization was carried out in a UVC1 cryochamber (Ted Pella Inc.) with a 360 nm lamp at -35°C for 48 h, and then at room temperature for a further 4 d. Thin sections were labelled as described above, and then stained with uranyl acetate and lead citrate.

RESULTS

Electrophoretic analysis of proteins extracted from *S. suis*

Cell surface components of *S. suis* were extracted by cell homogenization with a Sorvall Omnimixer. Analysis by urea-SDS-PAGE of the proteins present in the blended preparation after centrifugation revealed a major protein with a molecular mass of approximately 9 kDa (Fig. 1, lane 2). To determine whether the blending step could result in cell lysis, a viable count on blood agar plates was performed prior to and after blending. The results (6.6×10^7 c.f.u. ml^{-1} prior to and 5.6×10^7 c.f.u. ml^{-1} after blending) suggested that viability was not substantially affected by this treatment. Examination of the cells by light microscopy revealed that these cocci occurred singly or in pairs and that their structural integrity was preserved following blending (not shown). It was therefore unlikely that the high amount of 9 kDa

Fig. 1. Computer-assisted reproduction of SDS-urea PAGE. The gels were scanned using Deskscan II from Microsoft and the background was reduced using the software Photofinish. Lanes 1, 2 and 3 represent protein bands separated by SDS-PAGE and stained with Coomassie blue; lane 4 illustrates results from a Western blot experiment. Lane 1, molecular mass markers; lane 2, blended preparation obtained from *S. suis* 170B; lane 3, purified 9 kDa protein; lane 4, immunoblot of the purified 9 kDa *S. suis* protein using a rabbit anti-*S. salivarius* HPr antiserum.

Smu	MASKDFHIVA	ETGIHARPAT	LLVQTASKFA	SDITLDYK GK	40
Ssa	MASKDFHIVA	ETGIHARPAT	LLVQTASKFA	SDITLDYK GK	
Ssu	ASKDFHIVA	ETGIHARPAT	LLVQTASKFA	SDITLNYK	

Fig. 2. Amino acid sequence alignments of HPr proteins from *S. mutans* (Smu) and *S. salivarius* (Ssa) with the first 37 amino acids determined for the 9 kDa protein extracted from the cell surface of *S. suis* 170B (Ssu).

protein found in the blended preparation was a consequence of an enrichment caused by cell lysis during blending. This protein was further purified to homogeneity by treatment of the blended preparation with 6 M urea and filtration as described in Methods (Fig. 1, lane 3).

Characterization of the 9 kDa protein

A database search using the sequence of the first 37 amino acids determined for the protein extracted from *S. suis* revealed 95% identity with the N-terminal amino acid sequences of HPr proteins from *S. salivarius* (Gagnon *et al.*, 1993) and *S. mutans* (Boyd *et al.*, 1994) (Fig. 2). Apart from the lack of the N-terminal Met, the only difference observed between the *S. suis* protein and these streptococcal HPrs was the replacement of an aspartate at position 36 by an asparagine. The *S. suis* protein possessed the signature sequence required for the PEP-dependent phosphorylation of Gram-positive HPrs on histidine at position 15 (GIH¹⁵ARPAT) (Reizer *et al.*, 1993a).

The size and the N-terminal amino acid sequence of the protein isolated from the cell surface of *S. suis* 170B suggested that this protein might be an HPr. The identity of the protein was confirmed by three series of experiments demonstrating: (1) the reactivity of the protein with a polyclonal monospecific anti-HPr antibody; (2) the capacity to be phosphorylated at the expense of PEP and ATP, a characteristic trait of HPrs from Gram-positive bacteria (Reizer *et al.*, 1993b); and (3) its phosphocarrier activity in an *in vitro* PTS assay.

Western blotting demonstrated that the purified 9 kDa protein cross-reacted with an *S. salivarius* anti-HPr antiserum (Fig. 1, lane 4), indicating a structural relationship between the *S. suis* protein and the *S. salivarius* HPr. The cross-reactivity of the 9 kDa protein with an anti-HPr antiserum allowed us to demonstrate by crossed immunoelectrophoresis that the protein could be phosphorylated by PEP and ATP. We had already shown that this technique allows a clear separation of free HPr and HPr-P (Vadeboncoeur *et al.*, 1991a). HPr(His-P), produced by EI and PEP, and HPr(Ser-P), produced by a membrane-associated kinase and ATP, migrate to the same position. However, the heat stability of the different phosphate bonds provides a convenient way to identify these derivatives. The phosphoramidate bond [HPr(His-P)] is thermolabile, whereas the phosphomonoester bond [HPr(Ser-P)] is thermostable (Martensen, 1984). The *S. suis* protein was first incubated in the presence of purified EI and PEP. After the incubation period, a portion of the sample was boiled for 3 min before examination by

Fig. 3. Crossed immunoelectrophoresis analysis of *in vitro* phosphorylation assays with the 9 kDa protein extracted from *S. suis*. (a) 1, protein purified from *S. suis*; 2, *S. suis* protein after incubation for 60 min at room temperature with 2 mM PEP and 37.5 ng EI purified from *S. sanguis*; 3, same as 2, but boiled for 3 min before electrophoresis. (b) 1, 0.5 mg of the protein extracted from *S. suis*; 2, *S. suis* protein after incubation for 90 min at 37 °C in the presence of 2.3 µg of membrane proteins isolated from *S. salivarius* as a source of HPr kinase and 0.1 mM ATP; 3, same as 2, but boiled for 3 min before electrophoresis. Samples were probed with a rabbit anti-*S. salivarius* HPr antiserum (12 mg total serum proteins).

crossed immunoelectrophoresis. The results in Fig. 3(a) indicate that exposure of the *S. suis* protein to EI in the presence of PEP gives rise to a heat-labile phosphoprotein that cross-reacts with an anti-HPr antibody. The protein was also incubated with ATP and *S. salivarius* membrane fragments as a source of HPr kinase. Crossed immunoelectrophoresis of heated and unheated samples following incubation with ATP revealed that the *S. suis* protein could be phosphorylated at the expense of ATP to produce a thermostable phosphoprotein (Fig. 3b). Using purified *S. salivarius* HPr, we showed that the phosphorylated and free forms of the *S. suis* protein migrated in crossed immunoelectrophoresis as phospho- and free *S. salivarius* HPr (not shown).

Finally, we showed that the 9 kDa protein extracted from *S. suis* 170B was able to catalyse the PEP-dependent phosphorylation of a PTS sugar (fructose) in the presence of purified EI and membrane fragments isolated from fructose-grown cells of *S. salivarius* as a source of II^{Fru} . The activity obtained with the *S. suis* protein was, however, approximately fourfold lower than that obtained with purified *S. salivarius* HPr [259 ± 24 vs 1118 ± 145 nmol phosphorylated fructose (mg HPr) $^{-1}$ h $^{-1}$]. The fact that the *S. suis* protein was exposed to urea during its purification might explain this lower specific activity.

Location of the 9 kDa protein

The ultrastructural location of the 9 kDa protein was determined by immunogold electron microscopy using *S. salivarius* HPr antiserum. Experiments performed with whole cells showed that the protein was associated with material surrounding the cells (Fig. 4a). No labelling was detected with a pre-immune serum (Fig. 4b). Experiments with thin sections showed that the anti-HPr antibody preparation reacted with a protein that was distributed throughout the cytoplasm, with a significant proportion of the HPr located at the cell periphery (Fig. 4c). Western blot experiments carried out with crude as well as with membrane-free cellular extracts confirmed that the antibody reacted solely with a protein that migrated to the same position as HPr in SDS-PAGE (not shown; see also results below).

S. suis contains two forms of HPr

That *S. suis* contains the two forms of HPr previously reported in streptococci and lactococci (Robitaille *et al.*, 1991) was demonstrated by separating the cytoplasmic proteins of *S. suis* and *S. salivarius* by non-denaturing electrophoresis, transferring the proteins to a nitrocellulose membrane, and testing for the presence of HPr with an antibody directed against the *S. salivarius* HPr.

Fig. 4. Transmission electron micrographs of *S. suis* 170B. (a) Whole cells incubated sequentially with anti-HPr antibody and gold conjugate; (b) whole cells treated as in (a) but with pre-immune serum; (c) thin section treated as in (a). Bar, 200 nm.

Fig. 5. Western analysis with anti-HPr antibody. Membrane-free cell extracts containing 10 µg protein were separated electrophoretically under non-denaturing conditions, electrophoretically transferred to a nitrocellulose membrane and revealed with HPr antibody. Lane A, *S. suis*; lane B, *S. salivarius*.

The results shown in Fig. 5 indicate that *S. suis* (Fig. 5, lane A) and *S. salivarius* (Fig. 5, lane B) possess two proteins that react with the anti-HPr antibody, the slow-migrating form of the doublet corresponding to HPr-1 (HPr lacking the N-terminal Met) and the fast-migrating form corresponding to HPr-2 (HPr possessing the N-terminal Met) (Vadeboncoeur *et al.*, 1991b). The *S. suis* HPr migrated more slowly than the *S. salivarius* protein, indicating differences in the conformation and/or the amino acid composition of the two proteins. This is consistent with the observation that the aspartate at position 36 is replaced by an asparagine in the *S. suis* HPr (Fig. 2). The results in Fig. 5 also indicate that the anti-*S. salivarius* HPr antiserum did not react with any cytoplasmic protein other than HPr-1 and HPr-2.

DISCUSSION

The presence of a PTS has never been reported in *S. suis*. The presence in this species of two PTS proteins, HPr (this paper) and a IIA^{Man}-like protein (Pelletier *et al.*, 1995), suggests that *S. suis* possesses a functional PTS, which is consistent with reports that this transport system is commonly found in fermentative eubacteria (Dills *et al.*, 1980).

There is little doubt that the protein extracted from whole cells of *S. suis* was actually HPr. The evidence is as follows: (i) the *S. suis* protein showed homology over the first 35 N-terminal amino acid residues with the HPrs of *S. salivarius* and *S. mutans*, including the signature sequence for the site of PEP-dependent phosphorylation; (ii) it

cross-reacted with the *S. salivarius* polyclonal monospecific anti-HPr antiserum; (iii) it could be phosphorylated by EI at the expense of PEP, and by a membrane-associated kinase at the expense of ATP; and (iv) it possessed phosphocarrier activity when used as a source of HPr in an *in vitro* PTS assay.

HPr is usually found in the cytoplasm or associated with the inner surface of the cytoplasmic membrane (Saier *et al.*, 1982). Results of three independent studies, however, suggest that streptococcal HPr might be associated with the cell surface. Jenkinson (1989) reported the purification of HPr from *S. sanguis* using an extraction procedure designed to remove cell-surface proteins. Sutcliffe *et al.* (1993) demonstrated that a surface antigen of *S. mutans*, designated antigen D, is an HPr, and showed that it could be extracted from whole cells by HECAMEG and lauroyl sarcosinate, two detergents used to extract surface components (Russell, 1988). Finally, Gerlach *et al.* (1992) reported the purification of HPr from culture supernatants of *S. pyogenes*. Results reported in this paper, for the first time, provide convincing direct evidence that a portion of the cellular HPr is located at the cell surface in streptococci. This is suggested by the fact that the *S. suis* HPr is preferentially extracted following blending of the cells, a method used to extract cell surface appendages (Heckels & Virji, 1988) without causing significant lysis. The presence of HPr on the cell surface was confirmed by immunogold electron microscopy carried out with whole cells, demonstrating the association of HPr with material surrounding the cell.

We have previously shown that several streptococcal species, including *S. salivarius*, *S. thermophilus*, *S. mutans*, *S. sobrinus*, *S. rattus*, *S. sanguis* and *S. bovis*, which belong to four different phylogenetic clusters (Bentley *et al.*, 1991), possess two forms of HPr that can be distinguished by the absence (HPr-1) or the presence (HPr-2) of the N-terminal Met (Robitaille *et al.*, 1991; Vadeboncoeur *et al.*, 1991b). We have demonstrated in this study that this dimorphism also occurs in *S. suis*, a species that is phylogenetically separate from other streptococcal species (Bentley *et al.*, 1991), suggesting that this phenomenon is ubiquitous among the genus *Streptococcus*. Although both forms of HPr are found in crude cell extracts of streptococcal cells, only HPr-1 has been found associated with the external cell surface (this paper; Gerlach *et al.*, 1992; Jenkinson, 1989; Sutcliffe *et al.*, 1993). Whether there is a cause-effect relationship between the conditional removal of the N-terminal Met of HPr and its cellular location remains to be determined. Further research is also needed to unravel the function of extracellular HPr and to define the molecular mechanism of its surface localization. This latter aspect is particularly intriguing as the HPr protein has no sequence corresponding to previously described signal peptides (Simonen & Palva, 1993).

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