Properties of a Phosphocarrier Protein (HPr) Extracted from Intact Cells of *Streptococcus sanguis*

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Cells of *Streptococcus sanguis* strain Challis were incubated with sodium lauroylsarcosinate to extract surface proteins. A polypeptide of apparent molecular mass 16 kDa comprising about 12% of the extract was purified using anion-exchange chromatography. The polypeptide was shown to be a phosphocarrier protein (HPr) that could also be found in the soluble (cytoplasmic) fraction from cells broken by homogenization with glass beads. *In vivo* labelling of *S. sanguis* cells with $^{32}$P showed that the polypeptide carried a heat- and acid-stable phosphorylation and that during sucrose starvation the HPr became dephosphorylated. Antiserum raised to the *S. sanguis* HPr reacted on Western blots with HPrs from all oral streptococci tested, together with strains of *S. pyogenes* and *S. salivarius*, but not with HPrs from *S. faecalis* or *S. bovis*, nor with proteins from *Staphylococcus aureus*, *Bacillus subtilis*, *Actinomyces viscosus* and various lactobacilli. The *S. sanguis* HPr had a high content of alanine (17.2%) and was similar in overall amino acid composition to the HPrs from *S. mutans* and *S. salivarius*. The N-terminal residues (to 37) of the *S. sanguis* HPr showed strong sequence identity (82%) with the N-terminal sequence of *S. faecalis* HPr. It is suggested that HPr in *S. sanguis* is associated closely with the cytoplasmic membrane. Non-disruptive methods of removing cell-surface proteins from streptococci effect release of HPr and possibly other cytoplasmic components.

INTRODUCTION

*Streptococcus sanguis* is an early colonizer of the clean tooth surface (van Houte et al., 1970) and is a major streptococcal species in human dental plaque (Gibbons & van Houte, 1975; Gibbons, 1984). Together with other prominent oral streptococci such as *S. mutans* and *S. salivarius*, it transforms dietary sugars into organic acids that evoke dental caries (van Houte, 1980; Hamada & Slade, 1980), and also into the polysaccharide glucans and fructans (Hamada & Slade, 1980) that are integral components of dental plaque matrix. The metabolism of carbohydrates by oral streptococci is thus crucial to the processes of colonization, plaque formation and caries development.

The phosphotransferase system (PTS) catalyses the uptake of carbohydrates and their concomitant phosphorylation in both anaerobic and facultatively anaerobic bacteria (Robillard, 1982; Saier, 1989). The oral streptococci possess multiple transport systems for carbohydrates. *S. mutans* transports sucrose via two PTSs (Slee & Tanzer, 1982) and also by a non-PTS mechanism (Ellwood & Hamilton, 1982). There are two glucose PTSs in *S. mutans* (Vadeboncoeur & Trahan, 1983a), in *S. sanguis* (Vadeboncoeur & Trahan, 1983b) and in *S. salivarius* (Vadeboncoeur & Trahan, 1982). In each case the PTS requires phosphoenolpyruvate (PEP), cytoplasmic components Enzyme I (EI) and phosphocarrier protein (HPr), and the sugar-specific Enzyme II (EI$_{\text{sugar}}$) associated with the cytoplasmic membrane (Postma &

**Abbreviations:** HPr, phosphocarrier protein; PEP, phosphoenolpyruvate; PTS, phosphotransferase system; SLS, sodium lauroylsarcosinate.
Roseman, 1976). The phosphoryl group of PEP is sequentially transferred to EI, then to HPr and finally to sugar in the presence of the components of the sugar-specific EII complex. An additional phosphocarrier protein (EIII) is an essential regulatory component of the system in *Escherichia coli* (Saier, 1989) but the role of EIII-like proteins in Gram-positive bacteria is not established (Reizer et al., 1988; Saier, 1989). Recent evidence suggests a sucrose PTS in *S. mutans* may be EIII-independent (Sato et al., 1989). HPr together with EI also catalyses the PEP-dependent phosphorylation of the non-PTS protein dihydroxyacetone kinase in *S. faecalis* (Deutscher & Sauerwald, 1986) and this may function in the regulation of glycerol uptake and metabolism in streptococci.

Gram-positive HPrs have been isolated from *Staphylococcus aureus* (Simoni et al., 1973) and the following streptococci: *S. faecalis* (Deutscher et al., 1986), *S. lactis* (Kalbitzer et al., 1982), *S. mutans* (Mimura et al., 1984; Thibault & Vadeboncoeur, 1985), *S. pyogenes* (Deutscher & Saier, 1983) and *S. salivarius* (Vadeboncoeur et al., 1983). Where they have been compared they appear to be functionally similar (Rosch et al., 1981; Kalbitzer et al., 1982) and in at least two regions structurally similar (Reizer et al., 1988; Schnierow et al., 1989). The primary function of the histidyl phosphorylation (at N1 of the imidazole ring) in HPr is to drive uptake of sugars into cells (Postma & Roseman, 1976). Several Gram-positive HPrs can be additionally phosphorylated on a seryl residue (serine-46) by an ATP-dependent kinase (Reizer et al., 1984; Deutscher et al., 1986), and this may regulate the rate of carbohydrate transport into cells of *S. mutans* (Lodge & Jacobson, 1988) and *S. pyogenes* (Reizer et al., 1984).

In this paper I describe the purification of HPr from *Streptococcus sanguis* using initially an extraction procedure designed to remove cell-surface proteins. The properties of the *S. sanguis* HPr and features of its N-terminal amino acid sequence are compared with those of other HPrs from Gram-positive bacteria.

**METHODS**

*Chemicals.* Amino acids, vitamins, sugars, sodium lauroylsarcosinate (SLS), egg-white lysozyme (EC 3.2.1.17), mutanolysin and molecular mass marker proteins were purchased from Sigma. BSA Fraction V was obtained from Calbiochem; Schiff’s reagent, Folin reagent, SDS, acrylamide and other chemicals were from BDH.

*Radiochemicals.* Bovine γ-globulin (100000 I.U., specific activity 1250 I.U. mg−1) was obtained from Cambridge Radiochemicals and was purchased from Amersham.

*Bacteria.* The bacterial strains used together with their sources or references are as follows: *Streptococcus sanguis* strain 0B11 (Challis), laboratory stock; *S. sanguis* NCTC 7869 (Channon), *S. sanguis* NCTC 9124 (Wicky), *S. sanguis* FW 227 and *S. sanguis* FW 35 from J. Ranhand (National Institutes of Health, Bethesda, MD, USA); *S. sanguis* 12, *S. sanguis* 12na, *S. sanguis* FW 213 and *S. sanguis* G99 from M. Willcox (Institute for Dental Research, Sydney, Australia); *S. anginosus* NHI 1237, *S. mitior* (oralis) O67331 and *S. bovis* NHI 1234 from National Health Institute, Porirua, New Zealand; *S. faecalis* JH2-2 from D. B. Clewell (University of Michigan, Ann Arbor, MI, USA); *S. mutans* NCTC 10449 (serotype e), *S. cricetus* E49, *S. ratti* FA1, *S. fera* MT 8184, *S. sobrinus* B13, *S. mutans* MT703R (serotype e), *S. mutans* OMZ175 (serotype f), *S. sobrinus* 6715, *S. pyogenes* prototypes M4 and M52, *S. salivarius* OB66 (recent clinical isolate), *Lactobacillus brevis* 409, *L. cellobiosus* 411, *L. jensenii* 422 and *L. leichmannii* 431 from J. Tagg (University of Otago, Dunedin, New Zealand); *Actinomyces viscosus* T14V from J. Cisar (National Institute of Dental Research, Bethesda, MD, USA); *Bacillus subtilis* 168 and *Staphylococcus aureus* ATCC 6538, laboratory stocks. All culture stocks were stored as freeze-dried suspensions in horse serum (Difco).

*Media.* Streptococci and actinomyces were cultured on BHYN agar (Jenkinson, 1986) at 37 °C in a Gas-Pak (BBL) system. Liquid cultures were grown at 37 °C in screw-capped bottles or tubes as stationary cultures in BHY medium or in glucose defined medium (Jenkinson, 1986). Low phosphate medium contained tryptone (10 g l−1), NaCl (2 g l−1), MgCl2 (10 mm), HEPES (0.1m) and sucrose (50 mm), brought to pH 7.2 with NaOH. *B. subtilis* and *Staph. aureus* were cultured on BHYN agar and in BHY medium aerobically at 37 °C. Lactobacilli were grown in LCM containing tryptone (20 g l−1), peptone (10 g l−1), yeast extract (5 g l−1), glucose (2 g l−1) and lactose (3 g l−1), pH 5.5, anaerobically at 37 °C, except for *L. brevis* and *L. cellobiosus* which were grown at 30 °C.

*Preparation of extracts.* Exponential phase cells of *S. sanguis* Challis were extracted with 1% (w/v) SLS to remove surface proteins as previously described (Jenkinson, 1986). Briefly, a sample of culture (1 ml) at OD600 1.5 (about 0.8 mg equiv. dry wt cells ml−1) was centrifuged for 3 min to collect cells, the supernatant was discarded and the bacteria were washed with 0.15 m-NaCl (1 ml). To the washed cell pellet was added 0.1 ml 1% SLS in 10 mm-Tris/HCl (pH 7.5) containing 1 mm-Na2EDTA (TE buffer). The cells were vortex-mixed and after 20 min at room-temperature (20 °C) the suspension was again vortex-mixed, then centrifuged at 10000 g for 5 min at 20 °C; finally, a portion of the supernatant (0.08 ml) was transferred to a fresh microfuge tube and centrifuged as before.
The supernatant after this final centrifugation is referred to as the SLS-extract, and this was either prepared and subjected to electrophoresis as described below, or frozen at -23 °C. For purification of the 16 kDa polypeptide approximately 10 g equiv. dry wt cells were incubated with 1% SLS (200 ml) for 20 min at 12 °C with intermittent shaking. The bacteria were sedimented by centrifugation (8000 g, 10 min, 4 °C) and the supernatant was collected by decanting from the pellets through several layers of muslin. The supernatant was again centrifuged (10000 g, 20 min, 4 °C) and the clear supernatant (about 180 ml) was made to a final volume of 2 litres with 95% ethanol. The mixture was left for 16 h at -23 °C; the precipitate was then collected by centrifugation (10000 g, 20 min, 4 °C), suspended in deionized water (20 ml), dialysed against water (5 litres, 4 h, 4 °C) and freeze-dried. When reconstituted in water or appropriate buffer this was the starting material for purification of the 16 kDa polypeptide.

To extract proteins from bacteria other than S. sanguis cultures (10 ml) were centrifuged at 6000 g for 10 min at 20 °C; the cells were then suspended in TE buffer (10 ml), divided into two equal portions and recentrifuged. One cell pellet was incubated with 1% SLS (0.5 ml) as described above. The other pellet (about 3.5 mg equiv. dry wt cells) was suspended in TE buffer (0.5 ml) containing 0.15 M-NaCl, lysozyme (50 μg ml⁻¹) and mutanolysin (10 μg ml⁻¹). The suspension was incubated at 40 °C for 15 min, frozen at -20 °C for 1 h, and then thawed at 40 °C. SDS (20% w/v, 0.025 ml) was added together with 2-mercaptoethanol (final concn 0.1% v/v) and the suspension was heated at 70 °C for 5 min. The extract was then centrifuged (10000 g, 10 min, 20 °C); a portion (0.2 ml) of supernatant was removed, bromophenol blue (0.01%) in 70% (v/v) glycerol was added (0.02 ml) and the extract was subjected to electrophoresis (see below).

Cell fractionation. Bacteria harvested from 100 ml culture (about 50 mg equiv. dry wt) were suspended in 10 ml TE buffer containing 1 mM-PMSE, mixed with glass beads (7 ml, 0.10-0.11 mm diam.) and broken in a Braun homogenizer (Jenkinson, 1986). The broken cell suspension was subjected to several low-speed centrifugations to remove unbroken cells and debris (Jenkinson, 1986), and was then centrifuged at 40000 g for 45 min at 4 °C. The pellet from this centrifugation was termed the 'envelope fraction' and the supernatant the 'soluble fraction'.

SDS-PAGE. Samples were electrophoresed through 13% (w/v) acrylamide gels containing 0.1% SDS by the system of Laemmli & Favre (1973). Proteins were stained with silver nitrate (Merril et al., 1981) or with periodic acid/Schiff as described by Kapitany & Zebrowski (1973). Stained gels were scanned with a recording laser densitometer (LKB UltraScan) in which the pen deflection was proportional to the absorbance. Molecular masses of proteins were estimated from their distances of migration by reference to a plot relating migration distances for eight marker proteins to log molecular mass. The markers were α₂-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephosphate isomerase (26.6 kDa) and lysozyme (14.3 kDa); all except the last were pre-stained with Coomassie blue. Proteins were transferred from acrylamide gels to nitrocellulose by electroblotting (Towbin et al., 1979) in 25 mM-Tris, 192 mM-glycine, 20% (v/v) methanol, pH 8.3, at 17 V cm⁻¹ for 60 min (0.75 mm thick gels) or for 90 min (1.5 mm thick gels). These were times for optimum transfer of the 16 kDa polypeptide to the nitrocellulose; longer transfer times led to losses of the polypeptide from the membrane.

HPLC. Proteins were separated on a DEAE-5PW column (7.5 cm × 75 mm i.d.) containing TSK gel (Toyo Soda Co.). The protein mixture (about 5 mg ml⁻¹) in 10 mM-NaH₂PO₄/NaOH buffer, pH 7.0, was applied to the column and the column was eluted with 25 ml of the same buffer at 1 ml min⁻¹, zero pressure and at 15 °C. The eluate was monitored at a detection wavelength of 206 nm. Once unbound material had come off the column was washed (0.5 ml or 1.0 ml) were collected automatically and samples from each (0.02 ml) were mixed with extraction buffer containing SDS and 2-mercaptoethanol (Jenkinson, 1987) and subjected to SDS-PAGE as described above.

Antisera and immunodetection. Male New Zealand White rabbits (age 3 months) were immunized intramuscularly with approximately 100 μg protein in Freund's complete adjuvant. After 6 weeks they were given a booster of a further 50 μg protein (without adjuvant) in 0.15 M-NaCl and bled 6 d later. Two months later they were given a further booster and bled after 7 d. Western blots and dot-blot were incubated with antiserum diluted (40-60 ml) of increasing (NH₄)₂SO₄ concentration according to the stage of purification (see Results). Fractions (0.5 ml or 1.0 ml) were collected automatically and samples from each (0.02 ml) were mixed with extraction buffer containing SDS and 2-mercaptoethanol and loaded onto a DEAE-5PW column. The column was then eluted with 25 ml of the same buffer at 1 ml min⁻¹, zero pressure, and at 15 °C. The eluate was monitored at a detection wavelength of 206 nm. Once unbound material had come off the column was washed (0.5 ml or 1.0 ml) were collected automatically and samples from each (0.02 ml) were mixed with extraction buffer containing SDS and 2-mercaptoethanol (Jenkinson, 1987) and subjected to SDS-PAGE as described above.

HPLC. Proteins were separated on a DEAE-5PW column (75 cm × 75 mm i.d.) containing TSK gel (Toyo Soda Co.). The protein mixture (about 5 mg ml⁻¹) in 10 mM-NaH₂PO₄/NaOH buffer, pH 7.0, was applied to the column and the column was eluted with 25 ml of the same buffer at 1 ml min⁻¹, zero pressure and at 15 °C. The eluate was monitored at a detection wavelength of 206 nm. Once unbound material had come off the column was washed (0.5 ml or 1.0 ml) were collected automatically and samples from each (0.02 ml) were mixed with extraction buffer containing SDS and 2-mercaptoethanol (Jenkinson, 1987) and subjected to SDS-PAGE as described above.

Amino acid analysis. Samples (5-10 nmol) were hydrolysed in vacuo at 105 °C for 24 h with 6 M-HCl and analysed using a Waters Millipore Amino Acid Analyzer equipped with a sulphonated polystyrene cation exchange column (4.6 mm × 25 cm). The buffer system was a binary gradient of sodium citrate (6 mM, pH 3-1) and boric acid (39 mM, pH 9.8) containing 0.25 M-NaCl. Some samples were treated with performic acid before hydrolysis and these were used to determine cysteine content. No corrections were applied to the amino acid analyses to account for losses of serine and threonine during hydrolysis.

N-terminal sequence analysis. Primary sequence analysis was done with an Applied Biosystems Gas Phase Sequencer (470A with on line 120A HPLC) using standard ABI protocols.

In vivo labelling of cells with 32P. Cells of S. sanguis strain Challis were radioactively labelled with 32P, essentially as described by Lodge & Jacobson (1988). Bacteria were pre-grown in low-phosphate medium containing yeast.
exposed at the cell-surface. A prominent component of the extract, comprising about 15% of the total protein, was a polypeptide of apparent temperature (20 °C) extracts 20-30 polypeptides (Jenkinson, 1986), a number of which were proteins from the mixture were not obtained and this was thought to be due to protein-protein interactions occurring within the column. Purification of the polypeptide was achieved using HPLC with a DEAE-5PW (anionic) column to which the 16 kDa polypeptide bound in 10 mM-NaH2PO4/NaOH buffer, pH 7.0. Portions of SLS extract that had been precipitated from ethanol (see Methods) were applied to the column, and the bound proteins were eluted with (NH4)2SO4 in phosphate buffer as two broad peaks (Fig. 1). The 16 kDa polypeptide was included in both peaks but was better represented in peak I which also lacked a major polypeptide of approximate molecular mass 31 kDa (Fig. 1). The proteins in peak I from a similar column which was then eluted with a more shallow (NH4)2SO4 gradient (0-0.4 M, 50 ml). Fractions (1 ml) were collected, portions were mixed with SDS and 2-mercaptoethanol and analysed by SDS-PAGE to detect the 16 kDa polypeptide. The polypeptide was eluted at 0.1-0.15 M-(NH4)2SO4 in phosphate buffer as two broad peaks (Fig. 1). The 16 kDa polypeptide was hydrophobic and prominent in the detergent extract it was of interest to purify and characterize this polypeptide. Initial attempts to achieve part-purification from SLS extracted protein; this in turn was obtained from about 10 g equiv. dry wt cells. Since the 16 kDa polypeptide was estimated to contribute at least 0.3% of total cell protein in S. sanguis (Jenkinson, 1986) this represents a recovery during purification of 1 to 2%.

RESULTS

Extraction and purification of 16 kDa polypeptide

Incubating cells of S. sanguis strain OB11 (Challis) with 1% SLS for 20 min at room temperature (20 °C) extracts 20-30 polypeptides (Jenkinson, 1986), a number of which were exposed at the cell-surface. A prominent component of the extract, comprising about 15% of the total protein, was a polypeptide of apparent $M_r$ 16 kDa. This polypeptide was hydrophobic and bound to octyl-Sepharose CL-4B in the presence of 1 M-(NH4)2SO4 (Jenkinson, 1986). In view of its hydrophobic properties and prominence in the detergent extract it was of interest to purify and characterize this polypeptide. Initial attempts to achieve part-purification from SLS extracts using octyl-Sepharose with gradient elution were unsuccessful. Clear separations of proteins from the mixture were not obtained and this was thought to be due to protein–protein interactions occurring within the column. Purification of the polypeptide was achieved using HPLC with a DEAE-5PW (anionic) column to which the 16 kDa polypeptide bound in 10 mM-NaH2PO4/NaOH buffer, pH 7.0. Portions of SLS extract that had been precipitated from ethanol (see Methods) were applied to the column, and the bound proteins were eluted with (NH4)2SO4 in phosphate buffer as two broad peaks (Fig. 1). The 16 kDa polypeptide was included in both peaks but was better represented in peak I which also lacked a major polypeptide of approximate molecular mass 31 kDa (Fig. 1). The proteins in peak I from a number of separate runs were pooled, dialysed against water, freeze-dried and re-applied to a similar column which was then eluted with a (NH4)2SO4 gradient (0-0.6 M, 50 ml). Fractions (1 ml) were collected, portions were mixed with SDS and 2-mercaptoethanol and analysed by SDS-PAGE to detect the 16 kDa polypeptide. The polypeptide was eluted at 0.1-0.15 M-(NH4)2SO4 (Fig. 2) and fractions containing the polypeptide (Fig. 3) were again pooled, freeze-dried and combined with similar fractions from other runs. Finally, a preparation containing approximately 0.5 mg protein and enriched in 16 kDa polypeptide was applied to the same column and eluted with a more shallow (NH4)2SO4 gradient (0-0.4 M, 50 ml). Fractions that appeared to contain only the 16 kDa polypeptide (Fig. 3) were pooled, dialysed against water and freeze-dried. The purified preparation contained >99% 16 kDa polypeptide (Fig. 3) as estimated from densitometer tracings of silver-stained polyacrylamide gels. On overloaded gels a faint silver-staining band at approximately 80 kDa was visible in the preparation (not shown). The higher-molecular-mass material stained in Fig. 3, gel B, is not this band but was across all lanes and is an artefact of silver staining on this particular gel. The 16 kDa polypeptide preparations separated by SDS-PAGE did not stain for carbohydrate with periodic acid/Schiff reagent.

Approximately 0.25 mg purified 16 kDa polypeptide preparation was obtained from 50 mg SLS-extracted protein; this in turn was obtained from about 10 g equiv. dry wt cells. Since the 16 kDa polypeptide was estimated to contribute at least 0.3% of total cell protein in S. sanguis (Jenkinson, 1986) this represents a recovery during purification of 1 to 2%.
Properties of Streptococcus sanguis HPr

Fig. 1. Purification of 16 kDa polypeptide from S. sanguis strain Challis by HPLC. In the first stage, proteins in an SLS-extract (5 mg protein ml⁻¹ in phosphate buffer, pH 7·0) were applied to a DEAE-SPW column, and the bound material was eluted with 0·6 M-(NH₄)₂SO₄ in phosphate buffer. Two main peaks at A₂₅₀ were obtained, the first (peak I) contained the 16 kDa polypeptide separated from other major components present in the original extract. The inset shows portions of each fraction subjected to SDS-PAGE and stained with silver nitrate.

Production of antiserum to the 16 kDa polypeptide

Antibodies were raised in rabbits to a preparation of 16 kDa polypeptide similar on SDS-PAGE to that shown in Fig. 3. To check the reactivity of the antiserum SLS-extracted proteins from S. sanguis Challis were subjected to SDS-PAGE, and electroblotted onto nitrocellulose; the Western blots were then incubated with various dilutions of antiserum or with pre-immune serum. At a 1:1000 dilution the antiserum reacted almost exclusively with the 16 kDa polypeptide (Fig. 4). A faint reaction of the antiserum was observed with a larger polypeptide band (just visible in Fig. 4), presumably the same protein (approximate molecular mass 80 kDa) that was a minor contaminant in the original 16 kDa polypeptide preparation. There was no reaction of pre-immune serum with the blots (not shown).

The antiserum was used to determine the relative amounts of 16 kDa polypeptide in various subcellular fractions prepared from S. sanguis Challis. A cell suspension was broken with glass beads by vigorous homogenization and then fractionated into 'soluble' and 'envelope' fractions by centrifugation (see Methods). Portions of the fractions diluted 2-fold serially in TE buffer were vacuum dot-blotted onto nitrocellulose together with likewise serially diluted samples of
Fig. 2. Purification of 16 kDa polypeptide from S. sanguis strain Challis by HPLC. In the second stage pooled preparations enriched in 16 kDa polypeptide from peak I in Fig. 1 were applied to a DEAE-5PW column and the bound material was eluted with a linear gradient of 0–0.6 M-(NH₄)₂SO₄ in phosphate buffer. The arrow marks the start of the gradient.

Table 1. Proportion of the 16 kDa polypeptide antigen in various protein fractions from S. sanguis strain Challis

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Protein* (µg per 10 µl)</th>
<th>16 kDa antigen† (µg per 10 µl)</th>
<th>Percentage 16 kDa antigen (by wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total broken cells</td>
<td>17·1</td>
<td>0·56</td>
<td>3·3</td>
</tr>
<tr>
<td>'Soluble'</td>
<td>16·0</td>
<td>0·61</td>
<td>3·8</td>
</tr>
<tr>
<td>'Envelope'</td>
<td>23·0</td>
<td>0·13</td>
<td>0·56</td>
</tr>
<tr>
<td>SLS-extract</td>
<td>8·5</td>
<td>0·98</td>
<td>11·5</td>
</tr>
</tbody>
</table>

* Total protein determined by the Lowry method.
† Concen determined from dot-blots (see text).

unfractionated broken-cell suspension and of SLS extract. Samples of purified 16 kDa preparation containing known amounts of protein (in the range 1–100 ng) were also dot-blotted, and the nitrocellulose sheets were incubated with antiserum (1:1000) and ¹²⁵I-labelled Protein A. The dilutions of purified protein preparation were used to generate a plot relating intensity of autoradiograph signals to amount of purified 16 kDa polypeptide present. The concentration of 16 kDa polypeptide in each of the various cell fractions was then determined by reference to the plot. The results (Table 1) show that the polypeptide makes up about 4% of the soluble fraction from cells. It is also present in the cell-envelope fraction but the proportion relative to that in total cell protein is negligible (Table 1). Clearly SLS extraction provides a fraction that is particularly enriched in this protein (11·5%; Table 1). Since the soluble protein fraction contains about 80% of total cell protein then 80% or more of total 16 kDa polypeptide is present in the soluble (cytoplasmic) fraction after cell breakage.

A number of streptococcal species and other Gram-positive bacteria were tested for production of antigens cross-reacting with antiserum raised to the 16 kDa polypeptide from S. sanguis Challis. In these experiments cells were grown to early stationary phase in BHY (or appropriate) medium and then either extracted with SLS as described, or incubated with a mixture of lysozyme and mutanolysin to part-digest peptidoglycan and then heated at 70 °C with SDS and 2-mercaptoethanol. This additional extraction was included because of the possibility
that a cross-reacting antigen present in another organism may not be extracted with the SLS method. In fact, it was found for the streptococci that when cross-reacting antigen was present it was extracted as well, or better, with SLS than with hot SDS.

Polypeptides solubilized from cells by the two methods were separated by SDS-PAGE and blotted onto nitrocellulose; the blots were then reacted with antiserum to the 16 kDa polypeptide. In Fig. 5 the lower portions only (in the 10–20 kDa range) of the blots of proteins separated from the various bacteria are shown.

The 16 kDa antigen was produced by all strains of *S. sanguis* tested (Fig. 5). In addition to the strains used and shown in Fig. 5, SLS extracts of strains 12 and 12na (Morris *et al.*, 1987), strain FW213 (Fachon-Kalweit *et al.*, 1985) and strain G9B all contained the 16 kDa antigen. Extracts

<table>
<thead>
<tr>
<th>Fraction</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
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<td>kDa</td>
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<td></td>
<td></td>
<td></td>
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</tbody>
</table>

![SDS-PAGE patterns](image)
Fig. 4. Reaction of antiserum raised to the 16 kDa polypeptide with proteins extracted from S. sanguis strain Challis with SLS. Lane 1, SDS-PAGE pattern of proteins (stained with silver nitrate) extracted from cells; lane 2, autoradiograph of a corresponding Western blot reacted with antiserum (1:1000 dilution) and 125I-labelled Protein A. The sample contained about 8 µg protein. Positions of molecular mass markers are shown for reference.

Fig. 5. Western blot analysis of proteins separated by SDS-PAGE after extraction from cells of different streptococci with SLS (lanes 1–10, 5 µg protein each lane) or with SDS at 70 °C (lanes 11–23, 20 µg protein each lane) as described in the text. Blots were incubated with antiserum raised to the 16 kDa polypeptide (diluted 1:500) then with 125I-labelled Protein A and were autoradiographed. Lane 1, S. sanguis Challis; lane 2, S. sanguis Wicky; lane 3, S. sanguis FW227; lane 4, S. sanguis Channon; lane 5, S. sanguis FW35; lane 6, S. pyogenes M52; lane 7, S. anginosus; lane 8, S. mutans NCTC 10449; lane 9, Actinomyces viscosus; lane 10, Bacillus subtilis; lane 11, S. cricetus; lane 12, S. rattus; lane 13, S. ferus; lane 14, S. sobrinus B13; lane 15, S. mutans MT703R; lane 16, S. mutans OMZ175; lane 17, S. sobrinus 6715; lane 18, S. mitior (oralis); lane 19, S. pyogenes M52; lane 20, S. pyogenes M4; lane 21, S. anginosus; lane 22, S. salivarius; lane 23, S. sanguis Challis.

from some strains of S. sanguis appeared to contain less 16 kDa antigen than others. For example SLS extract of strain Wicky (NCTC 9124) contained about half the amount of antigen that extract from Challis did, as estimated from densitometer tracings of silver stained gels of separated proteins (not shown).

All the other oral streptococci tested (S. mutans, S. rattus, S. sobrinus, S. ferus and S. cricetus), S. salivarius (group K) and S. pyogenes (group A), produced an antigen of approximately 16 kDa
that reacted on Western blots with antiserum raised to the S. sanguis protein (Fig. 5). The antiserum did not react at 1:500 dilution on Western blots with proteins from S. faecalis or S. bovis (not shown). The exact mobilities of the cross-reactive 16-kDa-like antigens on the gels of the various streptococcal species were slightly different and quite characteristic. For example, the antigen from S. anginosus migrated to an apparent molecular mass of 16.5 kDa while those from S. mutans and S. sobrinus had apparent molecular masses of about 17 kDa. These differences can just be seen in Fig. 5 but were more apparent on silver-stained 12% polyacrylamide gels (not shown).

Antibodies to the 16 kDa polypeptide did not bind to Western blots of cell proteins from the following bacteria: Actinomyces viscosus, Bacillus subtilis, Lactobacillus brevis, L. cellobiosus, L. jensenii, L. leichmannii and Staph. aureus.

N-terminal sequence analysis

The purified 16 kDa polypeptide from S. sanguis Challis was subjected to automated Edman degradation, which gave unambiguous information on the sequence of the intact protein for 36 residues. The sequence was compared with the SwissProt protein sequence database and it was found to have 82% identity in a 34 amino acid overlap with HPr from S. faecalis, and 70% identity in a 26 amino acid overlap with the HPr from Staph. aureus. The N-terminal 36 residues of the S. sanguis 16 kDa protein are compared and aligned with the N-terminal sequences of the S. faecalis, Staph. aureus and B. subtilis HPrs in Fig. 6. The sequencing did not identify an N-terminal methionine residue in the S. sanguis sequence, but the sequences are aligned in Fig. 6 on the assumption that the N-terminal residue in the S. sanguis sequence is missing (see Discussion). The residues in the S. sanguis sequence are numbered accordingly, i.e. 2 to 37. Residues 13 to 20 (active centre region) are conserved in all the HPrs (Fig. 6), histidine-15 being the residue involved in phosphoryl group transfer (Simoni et al., 1973; Beyreuther et al., 1977). Hydrophobic residues either side of the active centre (residues 6 to 10, and 19 to 23) are also present in all proteins. The S. sanguis protein has seryl residues at positions 28 and 31, the latter serine-31 being common to the streptococcal and staphylococcal sequences. All proteins have a tyrosine residue at position 37 which may interact with histidine-15 (Reizer et al., 1988). The kinase-mediated phosphorylations that are observed to occur on Gram-positive bacterial HPrs (Reizer et al., 1984) occur on serine-46 which is part of a second conserved region of 20 amino acids in Gram-positive bacterial HPrs compared by Schnierow et al. (1989).

The major difference in the N-terminal sequence of the S. sanguis protein compared with the others is in the proportion of hydrophobic amino acids, particularly in the 11 residues 26 to 36. In S. sanguis HPr eight of these are hydrophobic amino acids and the region contains only one charged residue (aspartate-32). By contrast, the same region of the Staph. aureus protein contains only three hydrophobic amino acid residues and three charged residues (Fig. 6); the S. faecalis protein contains four hydrophobic residues in this part of the sequence.
Table 2. Amino acid composition of HPrs from S. sanguis, S. mutans, S. salivarius, S. faecalis and Staph. aureus

Amino acid compositions other than for S. sanguis HPr are directly from, or calculated from, the following references: S. mutans, Mimura et al. (1984); S. salivarius, Vadeboncoeure et al. (1983); S. faecalis, Deutscher et al. (1986); Staph. aureus, Simoni et al. (1973). ND, Not determined.

<table>
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<th>Amino acid</th>
<th>S. sanguis</th>
<th>S. mutans</th>
<th>S. salivarius</th>
<th>S. faecalis</th>
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Amino acid composition

The amino acid composition of the S. sanguis HPr is shown in Table 2. The protein is characterized by having a high content of Glx (glutamate + glutamine), Asx (aspartate + asparagine), glycine and alanine. Despite the high alanine content its polarity (47.5%) calculated by summing the mol% of Asp, Glu, Lys, Ser, Thr, Arg and His (Capaldi & Vanderkooi, 1972) is close to the mean value found for soluble proteins (47 ± 6%) and similar to the polarities calculated for the other HPrs in Table 2. The overall amino acid composition of the S. sanguis HPr is similar to those of the HPrs from S. mutans and S. salivarius (Table 2). The oral streptococcal HPrs differ in amino acid composition from the S. faecalis HPr, and more so from the Staph. aureus HPr, mainly in alanine content and in methionine. The oral streptococcal HPrs can be calculated to each have one methionine residue only and probably no cysteine (Table 2).

Starvation-induced dephosphorylation of S. sanguis HPr

The heat- and acid-stable phosphorylation of HPr in some Gram-positive bacteria may be involved in regulation of sugar phosphate accumulation. Lodge & Jacobson (1988) showed that in sucrose-grown cells of S. mutans containing phosphorylated HPr, the protein became dephosphorylated over a period of 2 h when the cells were starved of sucrose. A similar experiment was done with S. sanguis strain Challis cells to determine if the 16 kDa polypeptide was phosphorylated and if it became dephosphorylated on sucrose starvation. Cells of S. sanguis were grown in low-phosphate medium to mid-exponential phase, harvested by centrifugation and pre-labelled by suspension in one-tenth volume of sucrose low-phosphate medium containing 32P. After pre-labelling the cells were starved of sucrose for 2 h. Duplicate samples were removed at intervals in this period and extracted immediately either with 1% SLS as before, or boiled with 0.5% SDS and 0.1% 2-mercaptoethanol for 3 min. The samples were then subjected to SDS-PAGE and the gels were either stained with silver nitrate or fixed and autoradiographed for detection of radioactive bands. Cells that had been starved for 2 h showed a similar silver-stained profile of SLS-extracted proteins to that from cells not starved (Fig. 7,
Fig. 7. Phosphorylation of *S. sanguis* strain Challis HPr and dephosphorylation concomitant with sucrose starvation. Cells grown in sucrose-containing medium and labelled *in vivo* with $^{32}$P, were incubated for 2 h without sucrose. At time-zero (lanes 1 and 3) and after 2 h (lanes 2 and 4) samples were withdrawn, the cells extracted with SLS, samples subjected to SDS-PAGE and the gels were stained with silver nitrate (lanes 1 and 2) or autoradiographed (lanes 3 and 4). A further sample was taken at time-zero and extracted with SDS at 100 °C (lane 5, stained gel; lane 6, autoradiograph). Lanes 1–4 contained approximately 1 μg protein, lanes 5 and 6 contained approximately 3 μg protein. Positions of molecular mass markers are shown for reference, and the HPr (apparent molecular mass 16 kDa) is arrowed.

lanes 1 and 2). SLS extraction was particularly effective at isolating phosphorylated proteins. At time-zero, the 16 kDa band was phosphorylated (Fig. 7, lane 3) as well as proteins in the 20–24 kDa range and a prominent band at an apparent molecular mass of 31 kDa (Fig. 7). Over the 2 h starvation period the $^{32}$P was lost from the 16 kDa polypeptide (Fig. 7, lane 4) but there was little change in the labelling of other bands. The same patterns of labelling were observed irrespective of whether samples were boiled or not prior to SDS-PAGE, indicating that the phosphorylations were possibly P-serine, P-threonine or P-tyrosine, but not 1-P-histidine. Boiling cells in SDS extraction buffer solubilized much more protein from the cells (Fig. 7, lane 5). However, the 16 kDa polypeptide was not well extracted and the other phosphoproteins were not so well represented in these extracts (Fig. 7, lane 6). The radioactivity associated with the gel origin in Fig. 7, lane 6, indicated that some of the phosphorylated proteins had become complexed by the SDS treatment as high molecular mass insoluble material.

**DISCUSSION**

Components of the PTS, HPr, EI and EII have been isolated from Gram-positive bacterial cells after mechanical breakage usually with glass beads (Beyreuther et al., 1977; Mimura et al., 1984; Deutscher et al., 1986) or with alumina (Vadeboncoeur et al., 1983). Generally, HPr and EI have been purified from the soluble fraction (Mimura et al., 1984; Deutscher et al., 1986), whereas EII has been purified from the membrane fraction (Vadeboncoeur et al., 1983). In this paper it is shown that HPr can be quickly and easily extracted from intact cells of *S. sanguis* by incubating them at room temperature with a solution of detergent such as sodium laurylsarcosinate; other detergents such as Nonidet P-40 and Zwittergent 3:12 are also effective in extracting the protein (Jenkinson, 1986), but SDS was not as effective. The detergent extraction procedure was originally devised to release surface proteins from cells of *S. sanguis* in
the search for components conferring properties of hydrophobicity and co-aggregation with other oral bacteria (Jenkinson, 1986). Since HPr is an intracellular protein in other systems it is likely then that the SLS-extraction procedure, as well as removing surface proteins, also allows release of cytoplasmic and membrane-bound polypeptides. HPr in S. sanguis is particularly easily released. It is present in extracts of cells incubated with 2 mM-barbital buffer, pH 8.6 (1 h, 4 °C) (data not shown), a procedure that has been used to extract cell-surface salivary adhesin complex from S. sanguis (Lamont et al., 1988). The profile of proteins obtained with barbital-extraction is very similar to that obtained by SLS-extraction except that about one-tenth the amount of protein is removed (unpublished data).

Vectorial labelling experiments with lactoperoxidase and 125I-iodide have shown that S. sanguis cells have about 30 polypeptides that are iodinated and thus proposed to be exposed at the cell surface (Appelbaum & Rosan, 1984; Jenkinson, 1986). Under these conditions, which label only exposed tyrosyl (and possibly histidyl) residues of proteins, the 16 kDa polypeptide appeared to be iodinated (Jenkinson, 1986). In the light of identifying the 16 kDa polypeptide as HPr these previous experiments have two implications. First, it is likely that the labelling agents lactoperoxidase and 125I-iodide penetrate the streptococcal cell-wall layers and probably label membrane-bound components, i.e. label is not confined to proteins within or outside the cell-wall peptidoglycan. Second, it is likely that HPr is associated with the membrane or is part of a membrane-associated complex. There is evidence that the PTS complex in E. coli is functionally associated with the membrane (Saier et al., 1982) and EIIG, lac permease and HPr can form a membrane-bound complex (Osumi & Saier, 1982). In S. mutans all the PTS components were found to remain tightly associated with the membrane fraction of cells broken by sonication or French pressure cell lysis, and vigorous agitation with glass beads was found to be necessary to remove EI and HPr (Mimura et al., 1984). The implications above both for the structure of the wall layers and for the location of HPr in S. sanguis do not provide a simple explanation as to precisely how HPr can be surface-labelled by lactoperoxidase-catalysed iodination. Either a particularly reactive region of the protein is accessible at the cytoplasmic membrane or perhaps the protein becomes indirectly labelled as a result of another exposed component of the proposed complex being labelled. A further possibility is that there may be normally some secretion either of the 16 kDa polypeptide, or of a complex containing it, by the cells, or possibly leakage of the polypeptide occurred during the iodination procedure. No evidence has been obtained, however, to show that the 16 kDa polypeptide is secreted into the culture fluid during growth of cells (Jenkinson, 1986).

SLS treatment of S. sanguis cells extracts a number of phosphorylated proteins (Fig. 7) — a major component of molecular mass 31 kDa (which is not dephosphorylated during sucrose starvation) and a group of labelled proteins at 20–24 kDa (Fig. 7). This latter material stains particularly well with silver nitrate but not with Coomassie blue (not shown). This suggests that some of the 32P-containing material in the 20–24 kDa region may be associated with polysaccharide (see for example Emdur et al., 1974). SDS extraction of cells at 100 °C seems to complex phosphorylated material in a high molecular mass form (Fig. 7).

Antiserum to the S. sanguis HPr reacted with proteins of similar molecular mass in all the other oral streptococci, but not with S. faecalis, S. bovis nor B. subtilis. Antiserum to the Staph. aureus HPr did not cross-react with the HPr from B. subtilis (Reizer et al., 1984). Antiserum to the S. mutans HPr also reacted with other oral bacterial HPrs in double diffusion tests (Thibault & Vadeboncoeur, 1985). These authors showed that no precipitin lines were formed with B. subtilis and Staph. aureus HPrs and that spurs were formed with S. faecium HPr. Antibodies to the EI from S. salivarius cross-reacted with EI proteins from other oral streptococci (Vadeboncoeur et al., 1983) but not with S. faecalis or B. subtilis. Thus the non-specific components of the PTS, HPr and EI are antigenically very similar in streptococcal groups A, H, K and the oral group, but not so with group D.

The molecular masses of the HPrs from Staph. aureus, S. salivarius and S. mutans are 8.3 kDa (Reizer et al., 1988), 6.7 kDa (Vadeboncoeur et al., 1983) and about 8 kDa (Thibault & Vadeboncoeur, 1985), respectively. The S. salivarius and S. mutans proteins run anomalously on SDS-PAGE without urea present with apparent molecular masses of 13 kDa (Vadeboncoeur et
The HPrs from the oral streptococci differ from the *Staph. aureus* HPr mainly in their high alanine content. Six of the alanine residues in the *S. sanguis* HPr are within the N-terminal portion (up to residue 37). There is an extensive region of hydrophobic residues between residues 26 and 37 in the *S. sanguis* HPr which may explain why the protein attaches readily to octyl-Sepharose (Jenkinson, 1986). In addition it could have functional significance with respect to the suggestion that the protein may be part of a membrane-bound complex. The HPr as isolated and sequence lacked an N-terminal methionine. In view of other Gram-positive bacterial HPrs that have been sequenced having N-terminal methionines it seems likely that this residue has been removed from the *S. sanguis* protein by post-translational cleavage. However it is not possible to tell if this occurred in vivo or whether N-terminal cleavage occurred after extraction.

The N-terminal sequence of the 16 kDa polypeptide from *S. sanguis* is entirely consistent with its function as an HPr. The histidine-15 residue is part of an active site that has no predictable secondary structure (Beyreuther *et al.*, 1977) and which may protrude, with short hydrophobic segments either side of the active centre. Residues 2 to 11 are predicted to form a β-sheet and 24 to 34 a strong α-helix (Chou & Fasman, 1974), identical with the predictions for the *Staph. aureus* HPr (Beyreuther *et al.*, 1977). Residue arginine-17 is present in all HPrs examined (Rosch *et al.*, 1981) and the additional histidine residue in the *S. sanguis* protein (histidine-7) is also found in three other streptococcal HPrs (Rosch *et al.*, 1981). The *S. sanguis* HPr undergoes a heat- and acid-stable phosphorylation, and is dephosphorylated under conditions of sucrose starvation in a similar way to the *S. mutans* HPr (Lodge & Jacobson, 1988). The relatively high concentration of HPr protein present in *S. sanguis* cell extracts (Table 1) is entirely consistent with the estimated HPr concentration of 0·2 mM in *S. pyogenes* (Reizer *et al.*, 1984). Amounts of HPr varied with different strains of *S. sanguis* as did amounts produced by different strains of *S. mutans* (Thibault & Vadeboncoeur, 1985).

The SLS-extraction procedure was used originally to remove cell-surface proteins from *S. sanguis* (Jenkinson, 1986). Although the extraction did not appear to cause cell lysis the method does evidently lead to release from cells of cytoplasmic and/or membrane components. The method is selective, though, because the SDS-PAGE profiles of proteins extracted with SLS are completely different from those of either membrane or cytoplasmic protein extracts (Jenkinson, 1984), in order to elucidate the molecular details of the various sugar transport systems.

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Properties of Streptococcus sanguis HPr


