The Phosphoenolpyruvate:Mannose Phosphotransferase System of *Streptococcus salivarius*. Functional and Biochemical Characterization of $IIAB_L^{Man}$ and $IIAB_H^{Man}$†

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ABSTRACT: Previous studies have suggested that the phosphoenolpyruvate:mannose phosphotransferase system of *Streptococcus salivarius* consists of a nonphosphorylated enzyme II domain that functions in tandem with a separate enzymatic complex called III^{Man}. The III^{Man} complex is believed to be composed of two protein dimers with molecular masses of approximately 72 kDa. Analysis of these proteins by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate has indicated that one dimer is composed of two 38.9-kDa subunits called III_H^{Man}, and the other of two 35.2-kDa subunits called III_L^{Man}. This study was undertaken to determine (1) the number and nature of the phosphorylated residue(s) on III_H^{Man} and III_L^{Man} and the phosphorylation sequence allowing the transfer of the phosphoryl group from HPr(His∼P) to the mannose:PTS substrates; (2) whether III_H^{Man} and III_L^{Man} originate from two different genes or result from a posttranslational modification; and (3) whether these two proteins are involved in the phosphorylation of 2-deoxyglucose, a substrate of the phosphoenolpyruvate:mannose phosphotransferase system. We showed that both III_H^{Man} and III_L^{Man} were phosphorylated on two histidine residues. One phosphate bond was heat-labile (phosphorylation at the N^1 position of the imidazole ring), while the second was heat-resistant (phosphorylation at the N^3 position of the imidazole ring). The sequence of the first phosphorylation site was deduced by comparing the N-terminal amino acid sequence of both forms of III^{Man} with IIA domains of the EII−mannose family. The sequences of both forms were identical over the 15 first amino acids, that is, MIGIIIASHGKFAEG. The sequence of the second phosphorylation site was determined for III_L^{Man} as IHGQVATNxTP. Hence, III_H^{Man} and III_L^{Man} are PTS proteins of the IIAB type and should be renamed $IIAB_H^{Man}$ and $IIAB_L^{Man}$. $IIAB_H^{Man}$ and $IIAB_L^{Man}$ had different peptide profiles after digestion with proteases, indicating that these two proteins are encoded by two different genes. In vitro PEP-dependent phosphorylation assays conducted with a spontaneous mutant devoid of both forms of $IIAB^{Man}$ suggested that the phosphoenolpyruvate:mannose phosphotransferase system of *S. salivarius* is composed of an uncharacterized nonphosphorylated membrane component that works in tandem with $IIAB_L^{Man}$. The physiological functions of $IIAB_H^{Man}$ remain unknown.

In several bacteria, mainly obligate and facultative anaerobic species, the phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] simultaneously catalyses the transport and phosphorylation of mono- and disaccharides via a cascade of protein phosphorylation using PEP as the phosphate donor (*1*, *2*). The PTS is composed of two cytosolic proteins, enzyme I (EI) and HPr, which are involved in the transport of all PTS sugars, and of a family of sugar-specific EII complexes comprising three (and less frequently four) distinct regions called A, B, C, and D that were called domains even though they were not always fused to a single protein (*3*). EI and HPr, as well as domains IIA and IIB, undergo phosphorylation during sugar transport by the PTS. Domains IIC and IID are not phosphorylated and are always found in the cytoplasmic membrane. On the basis of sequence comparisons and the structural organization of the different domains, EIIs are grouped into six families: (1) the glucose−sucrose family, (2) the mannitol−fructose family, (3) the lactose−cellobiose family, (4) the mannose family, (5) the glucitol family, and (6) the galactitol family. Two EIIs belonging to the same family show at least 25% identity throughout their sequences while only local similarities are found among EIIs from different families (*2*, *3*).

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[1] Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHAPS, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DEPC, diethyl pyrocarbonate; 2-DG, 2-deoxyglucose; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; EI, enzyme I; EII, enzyme II complex; EDTA, ethylenediaminetetraacetic acid; HN, 100 mM Hepes (pH 7.5) + 125 mM NaF buffer; HPr, histidine-containing phosphocarrier protein; IPTG, isopropyl β−D-thiogalactopyranoside; NEM, *N*-ethylmaleimide; 2ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; PMSF, phenylmethylsulfonyl fluoride; PP, potassium phosphate buffer; PTH, phenylthiohydantoin; PTS, phosphoenolpyruvate:sugar phosphotransferase system; PVDF, poly(vinylidene difluoride); PVP, poly(vinylpyrrolidone); SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone.

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Transfer of the phosphoryl group from PEP to the incoming sugar always proceeds according to a common scheme, irrespective of the PTS involved. EI is first phosphorylated at the expense of PEP at the N^3 position of an histidine, while HPr is phosphorylated by EI∼P at the N^1 position of a conserved histidine residue at position 15. HPr(His∼P) transfers its phosphate group to the N^3 position of a histidine on the IIA domain of the EII complex. The second phosphorylation site of the EII complex, located on the IIB domain, is then phosphorylated by its IIA counterpart. The IIB domains of the glucose−sucrose, mannitol−fructose, lactose−cellobiose, glucitol, and galactitol EII families are phosphorylated on a cysteine, while the IIB domains of the EIIs belonging to the mannose family are phosphorylated at the N^1 position of an histidine (*3*).

The mannose:PTS of *Streptococcus salivarius* transports mannose, glucose, fructose, and the nonmetabolizable mannose analogue 2-DG (*4*). Previous studies have suggested that the enzyme II complex of this system forms a membrane module, which does not undergo phosphorylation and which functions in tandem with a separate enzymatic complex called III^{Man} (*4, 5*). From immunochemical, physiological, and biochemical studies, it was concluded that the III^{Man} complex is composed of two protein dimers with molecular masses of approximately 72 kDa. Analysis of these proteins by SDS−PAGE has indicated that one dimer, called III_H^{Man}, is composed of two 38.9-kDa subunits, while the other, called III_L^{Man}, is composed of two 35.2-kDa subunits (*4*). Both proteins undergo phosphorylation when membrane-free cellular extracts of *S. salivarius* are exposed to [^{32}P]PEP. This study was undertaken to determine (1) the number and nature of the phosphorylated residue(s) on III_H^{Man} and III_L^{Man}, (2) whether III_H^{Man} and III_L^{Man} originate from two different genes or whether III_L^{Man} results from a posttranslational modification of III_H^{Man}, and (3) whether these two forms are involved in the transport and phosphorylation of mannose:PTS substrates.

EXPERIMENTAL PROCEDURES

Organisms and Growth Conditions. S. salivarius ATCC 25975 was provided by Dr. I. R. Hamilton (University of Manitoba, Canada). Mutants A37 and G77 were isolated from *S. salivarius* ATCC 25975 by positive selection for resistance to either 0.5 (A37) or 5 mM (G77) 2-DG in the presence of 0.2% lactose or 0.2% galactose, respectively (*5, 6*). A37 is a III_L^{Man}-defective mutant while G77 lacks both forms of III^{Man}. Cells were grown at 37 °C in TYE broth, which contains 10 g of Tryptone, 5 g of yeast extract, 2.5 g of NaCl, and 2.5 g of disodium phosphate per liter. Glucose was sterilized by filtration (Millipore filter, 0.22 μm) and added aseptically to the medium to a final concentration of 0.5%. *Escherichia coli* XL-1 Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F′* proAB lacI^q*ZDM15 Tn10 (Tet^r)]*) was obtained from Stratagene.

Preparation of Cytoplasmic and Membrane Fractions. Cells were broken by grinding with alumina (*7*) in the presence of 10 mM PP buffer (pH 7.5) containing 0.1 mM PMSF, 14 mM 2ME, 1 μM pepstatin A, 1 μM leupeptin, and 1 mM EDTA. Cytoplasmic and membrane fractions were obtained by differential centrifugation as previously described (*4*). All steps were performed at 4 °C.

Partial Purification of the III^{Man} Complex. The cytoplasmic extract obtained from 72 L of culture of glucose-grown cells of *S. salivarius* 25975 was chromatographed on a HiLoad 16/10 Q Sepharose Fast Flow column (1.6 × 11 cm) (Pharmacia) equilibrated with 20 mM PP buffer (pH 7.5). The column was washed first with 10 volumes of 20 mM PP buffer (pH 7.5) and then with 5 volumes of the same buffer containing 0.1 M KCl. The column was then eluted with a 390-mL (0.1−0.6 M KCl) gradient in 20 mM PP buffer (pH 7.5) at a flow rate of 180 mL/h. The dimer forms of III_H^{Man} and III_L^{Man} were eluted in the same fractions at approximately 0.35 M KCl and were detected using anti-III_H^{Man} antibodies (these antibodies reacted with both forms of III^{Man}) (*4*). The fractions were pooled and dialyzed against 10 mM PP buffer (pH 7.5). The dialyzed sample was then applied to a Sephacryl S-200 column (2.6 × 66 cm) (Pharmacia) equilibrated with 10 mM PP buffer (pH 7.5) containing 0.1 M KCl. The column was washed with 400 mL of the same buffer at a flow rate of 150 mL/h. III_H^{Man} and III_L^{Man} were eluted in the same fractions, which were pooled and concentrated to 7.5−10 mg of protein/mL.

Purification of III_H^{Man} and III_L^{Man} by Two-Dimensional Gel Electrophoresis. III_H^{Man} and III_L^{Man} were purified to homogeneity by 2D-PAGE using the technique of O'Farrell (*8*) with modifications. A sample of partially purified III^{Man} (50 mL containing 700−800 μg of protein) was mixed with 37.5 mg of urea and 100 μL of a solution containing 5.7% (w/v) urea, 8% (w/v) CHAPS, 2% (v/v) 2ME, and 2% (v/v) of pH 5−6 ampholytes. The sample was applied to a gel (9 cm long and 3 mm in diameter) containing 4% (w/v) acrylamide, 5.5% (w/v) urea, 2% (w/v) CHAPS, and 2% (w/v) ampholytes (pH 5−6). The electrofocusing was carried out at room temperature at 300 V for 18 h followed by a 1-h run at 400 V. The second dimension was performed on a 10% acrylamide gel using 50 and 70 mA/gel during the migration in the stacking gel and the resolving gel, respectively. Under these conditions, III_H^{Man} migrated as a protein with a molecular mass of 40.7 kDa and a p*I* of 5.9, whereas III_L^{Man} migrated as a protein with a molecular mass of 34.7 kDa and a p*I* of 5.3.

N-Terminal Amino Acid Sequencing. After III_H^{Man} and III_L^{Man} were separated by 2D-PAGE, the proteins were transferred to a PVDF membrane using 10 mM CAPS buffer (pH 11.0) containing 10% methanol (v/v). After being stained with 0.1% Coomassie blue in 40% methanol/1% acetic acid for 1 min, the membrane was destained in distilled water and the spots corresponding to III_L^{Man} and III_H^{Man} (previously identified with anti-III_H^{Man} antibodies) were excised. N-Terminal amino acid sequences were determined by Edman degradation using a model 473A pulsed liquid-phase sequencer from Applied Biosystems. The sample was applied to a TFA-treated cartridge filter coated with 1.5 mg of Polybrene and 0.1 mg of NaCl. The PTH amino acid derivatives were identified by comparison with standards (PTH Analyzer standards, ABI) analyzed on-line prior to the sequence analysis.

Identification of the Phosphorylated Residues on III_H^{Man} and III_L^{Man}. [^{32}P]PEP was prepared according to the method of Mattoo and Waygood (*9*) using partially purified PEP carboxykinase from *E. coli* K12 HFr 3000 kindly provided by Dr. A. H. Goldie (University of Saskatchewan, Canada). The phosphorylation of proteins at the expense of [^{32}P]PEP

was carried out according to the procedure of Waygood et al. (*10*) with slight modifications. Samples were incubated for 15 min at room temperature in the presence of 0.025 mM [^{32}P]PEP (6.67 × 10^5 cpm/nmol), 1 μg of EI and 3 μg of HPr, both purified from *Streptococcus mutans* DR0001 (*11*), in a total volume of 60 μL. The reaction was stopped by the addition of 22.5 μL of a solution containing 188 mM Tris-HCl (pH 8.0), 6% (w/v) SDS, 30% (v/v) glycerol, 6% (v/v) 2ME, and 0.005% bromophenol blue. Heat sensitivity of the phosphorylated residues was determined by boiling samples for 1, 2, or 5 min. Boiled and unboiled samples were then loaded into the sample wells of polyacrylamide gels (16 × 16 cm) with a resolving gel containing 12.5% acrylamide and subjected to denaturing electrophoresis (SDS−PAGE). The gels were 1 mm thick and ran at 25 mA/gel for 4 h. After electrophoresis, the gels were immediately dried. To distinguish between acid-stable and acid-labile phosphate bonds, a duplicate of the polyacrylamide gel was fixed for 24 h in 16% TCA (with a change of solution after 12 h) before drying. To test for sensitivity of the phosphorylated residues to alkali, the phosphoproteins separated by SDS−PAGE were transferred electrophoretically to a PVDF membrane (0.45 μm) using 10 mM CAPS buffer (pH 11.0) containing 10% methanol (v/v). The membrane was then rinsed in 50 mM Tris/150 mM NaCl buffer (pH 7.4) for 10 min and then soaked in 1.0 N KOH for 2 h at 55 °C. The membrane was then neutralized by soaking in 50 mM Tris/150 mM NaCl (pH 7.4) for 1 min and then in 1.0 M Tris (pH 7.0) for 5 min. The membrane was finally rinsed twice with deionized water. Autoradiography of dried gels and membranes was performed at room temperature with exposure times of 18−24 h with Kodak X-ray film (X-Omat AR).

In Situ Peptide Mapping of Both Forms of III^{Man}. Partially purified III_H^{Man} and III_L^{Man} were separated by 2D-PAGE (*8*) as previously described, except that the gel used for electrofocusing was 1 mm in diameter. The second dimension was performed on a 10% acrylamide gel using a current of 15 and 25 mA/gel during migration in the stacking gel and the resolving gel, respectively. Peptide maps of III_L^{Man} and III_H^{Man} were obtained following the method developed by Cleveland (*12*). III_L^{Man} and III_H^{Man}, separated by 2D-PAGE, were excised from 1-mm gels stained with Coomassie blue, trimmed to 1 cm, and soaked with gentle swirling for 30 min in 10 mL of equilibration buffer containing 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 10% glycerol, 21.5 mM 2ME, and 1 mM EDTA. The gel pieces were then loaded into the wells of a 1.5-mm-thick acrylamide gel with a 3.5-cm stacking gel containing 4.5% acrylamide and a resolving gel containing 15% acrylamide. They were then overlaid with equilibration buffer containing 20% glycerol in order to fill the spaces around the pieces. Finally, 10 μL of the equilibration buffer and a given amount of the Glu-C endoproteinase (type XVII-B) from *Staphylococcus aureus* V8 (Sigma) (protein/protease ratio of 100:1) was added to the gel pieces, and electrophoresis was performed at 60 V during the migration in the stacking gel and at 40 mA/gel during the migration in the resolving gel. The temperature was maintained at 10 °C during the electrophoresis. The gel was then stained with silver nitrate (*13*).

Peptide Mapping by HPLC. After separation of III_H^{Man} and III_L^{Man} by 2D-PAGE, the proteins were transferred electrophoretically to nitrocellulose paper (0.45 μm) according to Towbin et al. (*14*). The nitrocellulose was briefly rinsed in deionized water, then stained with Ponceau Red (0.1% in 1% acetic acid) for 1 min and destained by washing for 1 min in 1% acetic acid. Spots corresponding to III_H^{Man} and III_L^{Man} were excised and briefly washed in Eppendorf tubes (15−30 s) in 1.0 mL of 200 μM NaOH. After a 1-min wash in deionized water, the spots were incubated for 30 min at 37 °C in 1.2 mL of 0.5% PVP-40 in 100 mM acetic acid. They were then washed extensively 5−10 times in 1.0 mL of deionized water and cut in to small squares (1 mm × 1 mm). The proteins were then eluted from the nitrocellulose membrane by incubation in 100 μL of 40% acetonitrile and 30% TFA for 2 h at room temperature with gentle, continuous shaking. After centrifugation at 10 000*g* for 10 min, the supernatant was removed and conserved and the pellet was resuspended in 100 μL of elution buffer and centrifuged at 10 000*g* for 5 min. The two supernatants were mixed and dried under vacuum. The proteins were then resuspended in 20 μL of 100 mM sodium phosphate buffer (pH 7.8), Glu-C protease type XVII-B from *S. aureus* V8 was added in a protein/protease ratio of 10:1, and the digestion was carried out for 20 h at 37 °C. Peptides generated were separated by HPLC (140A solvent delivery system from Applied Biosystems Inc). Peptides were isolated from a microbore C-18 reversed-phase column (2.1 × 220 mm, Applied Biosystems) using TFA at a flow rate of 0.2 mL/min with a linear (0 to 80% acetonitrile) gradient over 50 min. Peptides were detected at 215 nm using a 1000S diode array detector (Applied Biosystems).

Amino Acid Sequencing of the Phosphorylated Peptides of III$_L^{Man}$. When this work was undertaken, the genes encoding III_L^{Man} and III_H^{Man} had not been cloned. The gene coding for III_L^{Man}, *manL*, was recently cloned in pCRII from Invitrogen Corp. (Lortie, L.-A, Pelletier, M., Vadeboncoeur, C., and Frenette, M., unpublished results). III_L^{Man} was thus purified from *E. coli* XL-1 Blue transformed with plasmid pML17, which contains *manL* under the control of the *lac* promoter. Cells were grown at 37 °C with vigorous agitation in 500 mL of LB medium containing ampicillin (50 μg/mL) and tetracycline (10 μg/mL). When the culture reached an OD_{600} of 0.4, IPTG was added to a final concentration of 1 mM. After 2 h of induction, the cells were harvested and the pellet resuspended in 1/40 volume of 10 mM PP buffer (pH 7.5) containing 1 mM EDTA. The cells were then sonicated for two 15-s periods and the cytoplasmic extract was obtained as described (*4*). III_L^{Man} was partially purified as described in this paper. The recombinant III_L^{Man} (60 μg) was phosphorylated for 20 min at room temperature by [^{32}P]-PEP as described in this paper. The reaction was stopped and the sample loaded onto a 10% polyacrylamide gel and subjected to SDS−PAGE using the conditions described above, except that the samples were not boiled prior to loading. After electrophoresis, the gel was placed between two sheets of cellophane and autoradiography was performed at −80 °C for 18 h. The phosphorylated band corresponding to III_L^{Man} ∼P was cut from the gel and the protein electroeluted using the Centrilutor apparatus from Amicon. The electroelution was performed at 250 V for 2 h in a buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% SDS. The electroeluted sample was then concentrated to 50 μL by centrifugation at 5000*g*. A 2.0-mL volume of 50 mM Tris-HCl (pH 7.6) buffer containing 1 mM $CaCl_2$ was

then added and the sample concentrated once more to 50 μL. This procedure was repeated twice. TPCK-treated trypsin (4 μg) (Promega) was then added to electroeluted III_L^{Man} ~P (45 μg) in a total volume of 120 μL and the digestion was carried out for 20 h at 37 °C. The resulting peptides were separated by HPLC using a 140A solvent delivery system from Applied Biosystems Inc. Peptides were isolated from a microbore C-18 reversed-phase column (2.1 × 250 mm, Applied Biosystems) by washing with 20 mM PP buffer (pH 6.8) at a flow rate of 0.2 mL/min with a linear (0−85% acetonitrile) gradient over 90 min. The peptides were collected manually and the radioactivity was determined by measuring Cerenkov radiation. The radioactive peaks were then dried by vacuum evaporation and resuspended in 90 μL of 20 mM PP buffer (pH 6.8) containing 0.67 M urea and 0.45% Tween-20. The radioactive peptides were then reseparated on the same column at a flow rate of 0.2 mL/min using the same buffer system, except for the gradient, which was 0−80% acetonitrile over 90 min. The amino acid sequences were determined by Edman degradation as described in this paper.

Phosphorylation of III_H^{Man} and III_L^{Man} by HPr(His~P) and Interpeptide Phosphoryl Transfer. III_H^{Man} and III_L^{Man} as well as their phosphorylated ^{32}P-labeled derivatives were separated by SDS−PAGE using a 7.5% acrylamide gel according to Laemmli (*15*), except that samples were not boiled before loading. After determining the position of III_H^{Man} and III_L^{Man} (by a Western blot run in parallel and revealed with anti-III_H^{Man} antibodies), and III_H^{Man}~P and III_L^{Man}~P (by autoradiography), the bands corresponding to III_H^{Man} and III_L^{Man} and their phosphorylated forms were excised from the gel and proteins were electroeluted using the Centrilutor microelectroelutor from Amicon. The electroelution was performed at 250 V for 2 h in 25 mM Tris (pH 8.3) and 192 mM glycine buffer containing 0.1% SDS. The samples were then concentrated to 50 μL by centrifugation at 5000*g* with Centricon-10 concentrators (Amicon Inc.), resuspended in 2.0 mL of 100 mM Hepes buffer (pH 7.5) containing 125 mM NaF (HN buffer), and concentrated once more to 50 μL. [^{32}P]PEP-dependent phosphorylation assays were carried out as follows: the phosphorylation of III_H^{Man} and III_L^{Man} by HPr(His~P) was achieved by incubating either III_H^{Man} or III_L^{Man}, previously electroeluted from a polyacrylamide gel, for 30 min in the presence of 0.025 mM [^{32}P]PEP, 1 μg of EI, and 3 μg of HPr in a total volume of 60 μL. For the phosphorylation of III_L^{Man} by III_H^{Man} ~P and of III_H^{Man} by III_L^{Man} ~P, the nonphosphorylated (3−5 μg) form was incubated with the phosphorylated form (3−5 μg) for 30 min. Last, to determine whether the phosphorylation of III_H^{Man} and III_L^{Man} by HPr(His~P) was reversible, III_H^{Man} ~P and III_L^{Man} ~P (3−5 μg) were incubated separately with purified HPr for 30 min.

Treatment of III^{Man} with Chemical Reagents. (a) Modification by DEPC. For the selective modification of histidines, partially purified III^{Man} (100 μg of proteins) was incubated for 15 min at room temperature in 100 mM PP buffer (pH 6.5) (50 μL total volume) with 40 mM DEPC freshly prepared in 2% ethanol (*16*, *17*). The reaction was then stopped by the addition of 50 μL of HN buffer. Proteins were separated from the DEPC by gel filtration on a Bio-Gel P-6DG column (bed volume 10 mL) from Bio-Rad. The fractions containing the proteins were pooled and concentrated to 50 μL by centrifugation at 5000*g* using the Centrilutor microelectroelutor from Amicon. The volume of the sample was then adjusted to 2.0 mL with HN buffer and once more concentrated to 50 μL.

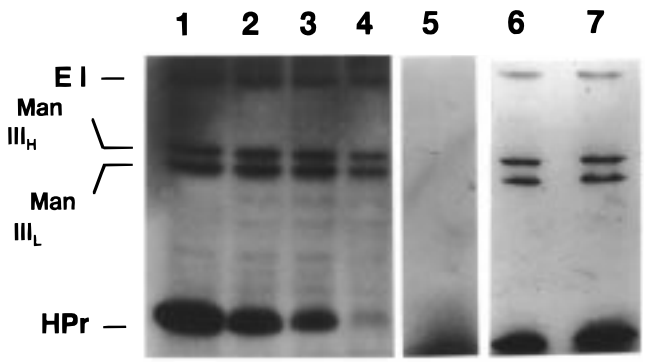

FIGURE 1: [^{32}P]PEP-dependent phosphorylation of III_H^{Man} and III_L^{Man} from *S. salivarius* ATCC 25975 using purified EI (1 μg) and HPr (3 μg) and [^{32}P]PEP (0.025 mM, 6.67 × 10^5 cpm/nmol). Experiments were carried out with partially purified III^{Man} complex (containing both forms of III^{Man}) (100 μg of total proteins). Lanes: (1) no treatment after phosphorylation; (2−4) samples boiled for 1, 2, and 5 min, respectively, after phosphorylation; (5) gel fixed in 16% TCA for 24 h. Results presented in lanes 6 and 7 were obtained after transfer to a PVDF membrane following electrophoresis of the phosphorylated proteins. (6) No treatment after transfer; (7) treatment with 1.0 N KOH for 2 h at 55 °C.

(b) Modification by NEM. For the selective modification of cysteine residues, partially purified III^{Man} (100 μg of proteins) was incubated in 0.24 M PP buffer (pH 7.0) containing 5% glycerol (total volume of 50 μL) with 30 mM NEM freshly prepared in reaction buffer (*18*). The sample was briefly flushed with nitrogen, sealed, and incubated for 4 h at room temperature. The reaction was then stopped by the addition of 50 μL of HN buffer. Proteins were separated from the NEM by gel filtration on a Bio-Gel P-6DG column (bed volume 10 mL) from Bio-Rad and were then concentrated using the procedure described above.

PTS Assay. The reaction mixture (600 μL) contained 50 mM sodium phosphate (pH 7.0), 4 mM $MgCl_2$, 2 mM PEP, 5 mM 2ME, 10 mM NaF, 10 mM ^{14}C-labeled 2-DG (0.1 μCi/μmol), 2 μg of EI and 20 μg of HPr purified from *S. mutans* DR0001, 200 μg of cytoplasmic proteins, and 200 μg of membrane proteins as a source of EII^{Man} permease. In some experiments, 18 μg of partially purified III_L^{Man} was added. After a 15-min preincubation at 37 °C, labeled 2-DG was added and the reaction was allowed to continue for 30, 60, and 120 min at 37 °C. Under these conditions, the reaction measured with membrane and cytoplasmic fractions of *S. salivarius* ATCC 25975 was linear with time for at least 2 h. The amount of phosphorylated 2-DG produced was determined as previously described (*7*).

RESULTS

Biochemical Properties of the Phosphorylated Residues of Phospho-III^{Man}. Incubation of a partially purified preparation of III^{Man} (which contains III_H^{Man} and III_L^{Man}) with EI, HPr, and [^{32}P]PEP resulted in the phosphorylation of four major proteins: EI, III_H^{Man}, III_L^{Man}, and HPr (Figure 1, lane 1). A number of experiments were carried out to determine the nature of the phosphorylated residues in III_H^{Man} and III_L^{Man} based on the fact that (1) phosphoramidates are

unstable under acidic conditions but relatively base-stable (*19*, *20*) and (2) N^1-phosphohistidines are thermolabile while N^3-phosphohistidines are heat-stable (*21*). To determine whether the phosphoryl bound of phospho-III^{Man} proteins were resistant to or unstable in acidic conditions, we immersed a polyacrylamide gel containing the phosphoproteins in a 16% TCA solution for 24 h. This treatment caused, as expected, complete dephosphorylation of EI and HPr but also dephosphorylated both forms of III^{Man} (Figure 1, lane 5). Conversely, treatment with 1.0 N KOH had no effect on these phosphate bonds (Figure 1, lanes 6−7). These properties are characteristic of *N*-phosphate bonds found in phospholysine, phosphoarginine, and phosphohistidine (*19*, *20*). As all known PTS proteins are phosphorylated on histidine or cysteine residues (*2*), we concluded that both forms of III^{Man} were phosphorylated at least on one histidine residue. To discriminate between phosphorylation at the N^1 and the N^3 positions of the imidazole ring of the histidine, we heated samples containing the phosphoproteins at 100 °C during 1, 2, and 5 min prior to electrophoresis. As expected, this treatment was without effect on phospho-EI (phosphorylated at N^1 position), while it caused gradual dephosphorylation of HPr (phosphorylated at N^3 position), which was almost completely dephosphorylated after 5 min of boiling (Figure 1. lanes 2−4). The phosphate groups linked to the III^{Man} proteins were partly lost after 5 min of boiling. The extent of loss, determined by computer-assisted densitometry, was estimated to be approximately 50%. The results suggested that each form of III^{Man} could be phosphorylated on two histidine residues giving rise to two phosphoramidate bounds that differed in their heat resistance.

We studied the effect of DEPC on the phosphorylation of III^{Man} as this compound inhibits histidine phosphorylation. We observed that the incubation of partially purified III^{Man} with 40 mM DEPC prevented the phosphorylation of both forms of III^{Man} (not shown). This result indicated that at least one histidine residue on III$_L^{Man}$ and/or III$_H^{Man}$ underwent phosphorylation when both forms were incubated together with HPr(His∼P) and that, in the absence of this reaction, no other phosphorylation could occur. Treatment of the sample with NEM did not prevent or reduce the phosphorylation of III$_H^{Man}$ or III$_L^{Man}$ (not shown), indicating that phosphorylation of these proteins by HPr(His∼P) does not result in the formation of phosphocysteine.

HPr(His∼P) Phosphorylated Both Forms of III^{Man}. The experiments reported above were carried out with partially purified III^{Man} preparations containing III$_H^{Man}$ and III$_L^{Man}$. The results suggested that both forms of III^{Man} possessed two phosphorylation sites, a characteristic of PTS IIAB proteins. This implied that both forms of III^{Man} could be phosphorylated directly by HPr(His∼P). To confirm this hypothesis, we performed phosphorylation experiments using III$_H^{Man}$ and III$_L^{Man}$ purified by SDS−PAGE as described in Experimental Procedures. The results indicated that both proteins were phosphorylated when incubated separately in the presence of purified EI, HPr, and [^{32}P]PEP (Figure 2, lanes 3 and 4). The phosphorylation reaction was reversible as free HPr could be phosphorylated by III$_H^{Man}$∼P and III$_L^{Man}$∼P (Figure 2, lanes 9−10). These results showed that both III$_H^{Man}$ and III$_L^{Man}$ possess a IIA domain.

Identification of a IIA Domain in III$_L^{Man}$ and III$_H^{Man}$. Samples of III$_L^{Man}$ and III$_H^{Man}$ purified by two-dimensional gel electrophoresis were used for N-terminal amino acid sequencing. III$_L^{Man}$ and III$_H^{Man}$ showed 100% identity over the first 15 amino acids (Figure 3A). Comparison of these sequences with the N-terminal sequences of other IIA domains in the mannose EII family shows that three amino acid residues are absolutely conserved (I, H, G), including the histidine at position 9 in the *S. salivarius* proteins, which has been shown to be phosphorylated in the IIA^{Man} domain of *E. coli* (*22*). The similarities vary from 50% with the IIA^{Man} domain of *Lactobacillus curvatus* to 73% with that of *E. coli*. In contrast, comparison of N-terminal amino acid sequences of III$_H^{Man}$ and III$_L^{Man}$ with IIA domains of other enzyme II families did not reveal any striking similarities (not shown). These results suggested that (1) III$_H^{Man}$ and III$_L^{Man}$ are proteins of the mannose family and (2) the N-terminal sequences of these proteins are part of IIA domains containing the histidine residue (His 9) that receives the phosphate from HPr(His∼P).

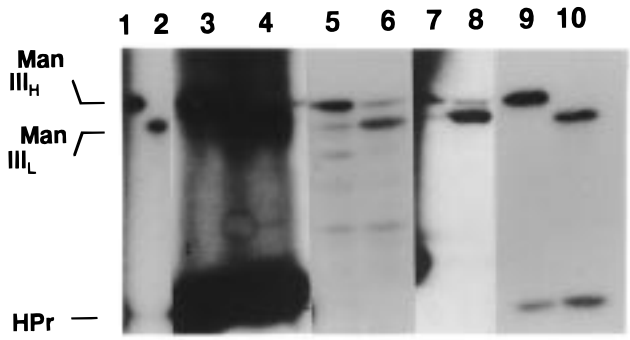

FIGURE 2: [^{32}P]PEP-dependent phosphorylation of the mannose: PTS proteins of *S. salivarius* ATCC 25975. The upper part of the gel containing phospho-EI is not shown. Lanes: (1) ^{32}P-labeled III$_H^{Man}$∼P (1 μg) purified by 1D SDS−PAGE; (2) ^{32}P-labeled III$_L^{Man}$∼P (1 μg) purified by 1D SDS−PAGE; (3) phosphoproteins produced following incubation of purified III$_H^{Man}$ (3−5 μg) with EI (1 μg), HPr (1 μg), and [^{32}P]PEP (0.025 mM, 6.67 × 10^5 cpm/nmol); (4) phosphoproteins produced following incubation of purified III$_L^{Man}$ (3−5 μg) with EI (1 μg), HPr (1 μg), and [^{32}P]PEP (0.025 mM, 6.67 × 10^5 cpm/nmol); (5) phosphoproteins produced following incubation of partially purified III^{Man} (containing both forms of III^{Man}) (100 μg of total proteins) with purified, ^{32}P-labeled III$_H^{Man}$∼P (3−5 μg); (6) phosphoproteins produced following incubation of partially purified III^{Man} (containing both forms of III^{Man}) (100 μg of total proteins) with purified, ^{32}P-labeled III$_L^{Man}$∼P (3−5 μg); (7) phosphoproteins produced following incubation of purified III$_L^{Man}$ (3−5 μg) with purified ^{32}P-labeled III$_H^{Man}$∼P (3−5 μg); (8) phosphoproteins produced following incubation of purified III$_H^{Man}$ (3−5 μg) with purified ^{32}P-labeled III$_L^{Man}$∼P (3−5 μg); (9) phosphoproteins produced following incubation of purified HPr (3 μg) with purified, ^{32}P-labeled III$_H^{Man}$∼P (3−5 μg); (10) phosphoproteins produced following incubation of purified HPr (3 μg) with purified, ^{32}P-labeled III$_L^{Man}$∼P (3−5 μg).

Identification of a Second Phosphorylation Site in III$_L^{Man}$. The gene coding for III$_L^{Man}$ has been recently cloned and expressed in *E. coli* (Lortie, L. A., Pelletier, M., Vadeboncoeur, C., and Frenette, M., unpublished results). This allowed us to obtain sufficient amounts of purified III$_L^{Man}$ to identify the second phosphorylation site. Purified III$_L^{Man}$ was phosphorylated with [^{32}P]PEP and digested by trypsin as described in Experimental Procedures. HPLC separation allowed the isolation of a radioactive peptide with the following sequence: LHGQVATN*x*TP, where *x* represents a residue destroyed during the Edman degradation. This sequence was very similar to the region around the phosphorylation site of other IIB domains that are members of

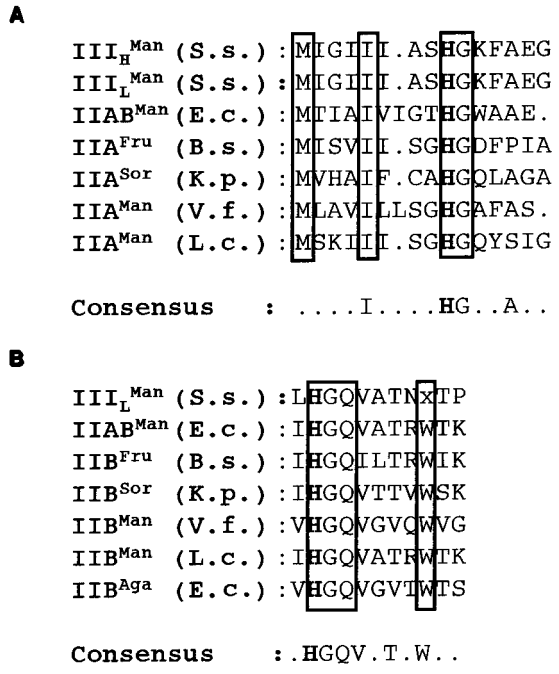

FIGURE 3: (A) Alignment of the N-terminal sequences of III$_H^{Man}$ and III$_L^{Man}$ with the IIA domains of the mannose EII family. The first 15 amino acids of III$_H^{Man}$ and III$_L^{Man}$ from *S. salivarius* (S.s.) were compared with those of IIA^{Fru} from *Bacillus subtilis* (B.s.) (*31*), IIA^{Sor} from *Klebsiella pneumoniae* (K.p.) (*32*), IIA^{Man} from *Vibrio furnissii* (V.f.) (*33*), IIA^{Man} from *Lactobacillus curvatus* (L.c.) (*34*), and IIAB^{Man} from *Escherichia coli* (E.c.) (*22*). (B) Alignment of the phosphorylated peptide isolated from P∼III$_L^{Man}$ with the phosphorylation site of the IIB domains of the mannose class of PTS proteins. The sequence of the phosphorylated peptide isolated from P∼III$_L^{Man}$ was compared with IIAB^{Man} from E.c. (*22*), IIB^{Fru} from *B. subtilis* (B.s.) (*31*), IIB^{Sor} from *K. pneumoniae* (K.p.) (*32*), IIB^{Man} from *L. curvatus* (L.c.) *34*, and IIB^{Aga} from *E. coli* (E.c.) (*35*). Sequences were obtained by Edman degradation as described in Experimental Procedures. Sequence comparisons were performed using the GCG sequence analysis package (*36*). Identical residues are boxed. Residues conserved in at least five sequences are presented in the consensus sequences. The presumed phosphorylated histidines are in boldface characters.

the mannose family (Figure 3B). If we assume that the amino acid destroyed during Edman degradation is a tryptophan residue, the data show that four amino acid residues (H, G, Q, W) are strictly conserved, including the histidine that undergoes phosphorylation (*22*). Three residues are partially conserved: the residue preceding the histidine may be isoleucine or valine; the residue following the glutamine (Q) may be valine or isoleucine; and the second amino acid preceding the conserved tryptophan residue may be threonine or valine. The sequence of the III$_L^{Man}$ peptide that was phosphorylated shares 81.8% similarity with the region around the phosphorylated histidine of *E. coli* IIB^{Man}. These results confirmed that III$_L^{Man}$ could be phosphorylated on two histidine residues. As we were unable to purify large amounts of III$_H^{Man}$, we could not repeat these experiments with this protein. However, the diagnostic tests reported in this paper have suggested that this protein could also be phosphorylated on two histidine residues. Hence, III$_H^{Man}$ and III$_L^{Man}$ are proteins of the IIAB type according to the nomenclature proposed by Saier and Reizer (*23*). They will be designated, hereafter, IIAB$_H^{Man}$ and IIAB$_L$Man.

Phosphotransfer between IIAB$_H^{Man}$ and IIAB$_L^{Man}$. To determine whether phosphotransfer can occur between the two forms of IIAB^{Man}, we incubated a partially purified preparation of IIAB^{Man} with either purified ^{32}P-labeled IIAB$_H^{Man}$∼P or ^{32}P-labeled IIAB$_L^{Man}$∼P. As shown in Figure 2 (lane 5), incubation in the presence of ^{32}P-labeled IIAB$_H^{Man}$∼P resulted in the phosphorylation of IIAB$_L^{Man}$ as well as three other proteins with molecular masses of 20, 25, and 30 kDa. Similarly, incubation of a partially purified preparation of IIAB^{Man} with ^{32}P-labeled IIAB$_L^{Man}$∼P resulted in the phosphorylation of IIAB$_H^{Man}$ and a protein with a molecular mass of 20 kDa (Figure 2, lane 6). Phosphotransfer between the two forms of IIAB^{Man} was also observed using purified unphosphorylated IIAB$_H^{Man}$ and IIAB$_L^{Man}$ instead of a partially purified preparation of IIAB^{Man} (Figure 2, lanes 7 and 8), indicating that this interpeptide phosphotransfer was not catalyzed by other proteins present in the partially purified fraction of IIAB^{Man}.

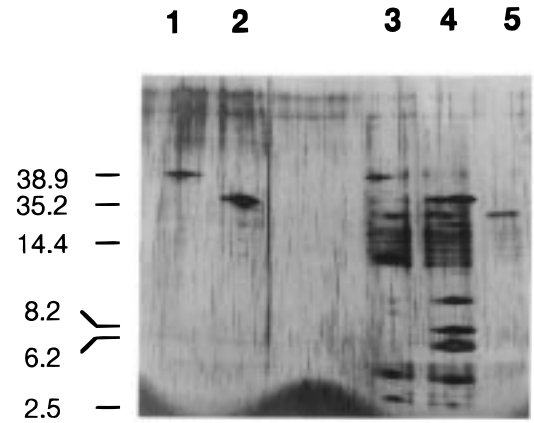

FIGURE 4: In situ peptide mapping of III$_H^{Man}$ and III$_L^{Man}$ using type XVII-B Glu-C protease from *S. aureus* V8. Proteins and peptides were separated on a 15% acrylamide gel and stained with silver nitrate. Lanes: (1) undigested III$_H^{Man}$ (3 μg); (2) undigested III$_L^{Man}$ (3 μg); (3) III$_H^{Man}$ (3 μg) + protease (0.03 μg); (4) III$_L^{Man}$ (3 μg) + protease (0.03 μg); (5) type XVII-B Glu-C protease from *S. aureus* V8 (0.03 μg).

IIAB$_H^{Man}$ and IIAB$_L^{Man}$ Are Encoded by Two Different Genes. Several results suggested that IIAB$_H^{Man}$ and IIAB$_L^{Man}$ are very similar proteins: they migrated together on different types of chromatography columns (*4*), both proteins react with anti-IIAB$_H^{Man}$ and anti-IIAB$_L^{Man}$, and their N-terminal amino acid sequences were 100% identical over at least the first 15 amino acid residues (Figure 3A). To determine whether these two proteins originate from different genes or whether IIAB$_L^{Man}$ is derived from limited proteolysis of IIAB$_H^{Man}$, we determined the fingerprint of the proteins after partial digestion with type XVII-B Glu-C protease from *S. aureus* V8 (*24*). Figure 4 (lanes 3 and 4) shows that the peptide profiles of IIAB$_H^{Man}$ and IIAB$_L^{Man}$ display several peptides with identical molecular weights, confirming the structural resemblance between these two PTS proteins. However, digestion of IIAB$_L^{Man}$ (the low-molecular-weight form) produced peptides that were not found on the peptide map of IIAB$_H^{Man}$, indicating that IIAB$_L^{Man}$ does not originate from a proteolytic cleavage of IIAB$_H^{Man}$. To confirm this result, we digested both proteins with *S. aureus* Glu-C protease and separated the resulting peptides by HPLC. We found that complete digestion of IIAB$_H^{Man}$ and IIAB$_L^{Man}$ generated several peptides common to both proteins but also gave rise to peptides characteristic of each form (Figure 5).

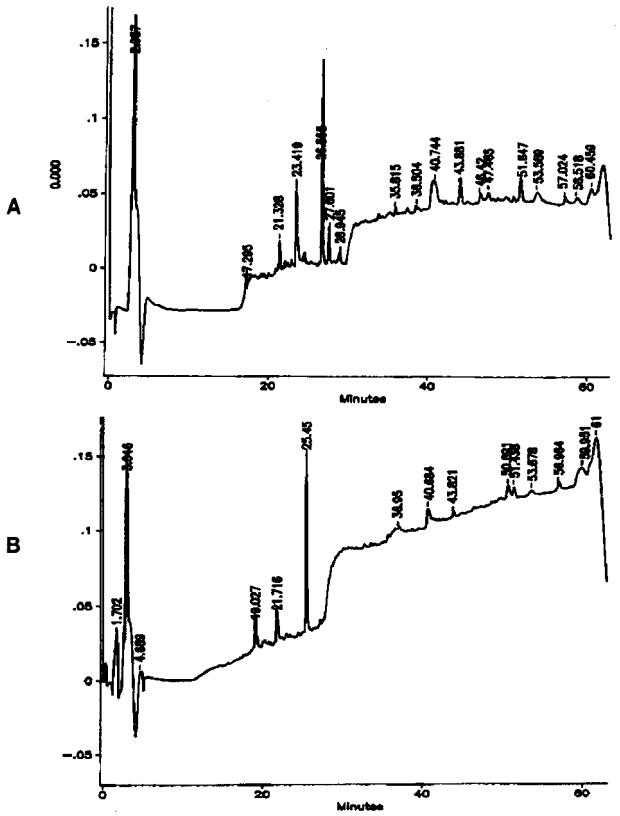

FIGURE 5: Peptides generated by digestion of III_H^{Man} and III_L^{Man} using protease XVII-B from *S. aureus* V8. The proteins (5 μg) were incubated in the presence of the protease (0.5 μg) for 20 h at 37 °C. The peptides were separated by HPLC (140A solvent delivery system from Applied Biosystems Inc.) on a microbore C-18 reversed-phase column (2.1 × 220 mm), at a flow rate of 0.2 mL/min with a linear (0−80% acetonitrile)gradient over 50 min. Peptides were detected at 215 nm with a 1000S diode array detector (Applied Biosystems Inc.). (A) Peptides generated with III_H^{Man}; (B) peptides generated with III_L^{Man}. The numbers on the figure indicate the elution time of the peptides.

Involvement of $IIAB_H^{Man}$ and $IIAB_L^{Man}$ in the Transport of Mannose:PTS Substrates. Our results have indicated that the proteins previously designated III_H^{Man} and III_L^{Man} are two distinct IIAB proteins. This raises the question of whether both proteins are components of the mannose:PTS in *S. salivarius*. In an attempt to answer this question, we carried out in vitro complementation PTS assays using mutant G77, a strain devoid of $IIAB_H^{Man}$ and $IIAB_L^{Man}$ (*6*) (Figure 6). Incubation of membranes and a membrane-free cellular extract of this mutant with EI, HPr, PEP, and ^{14}C-labeled 2-DG, a substrate of the mannose:PTS, resulted in a basal production of 2-DG-phosphate (first bar of Figure 6). When we replaced the membrane-free cellular extract of mutant G77 by a membrane-free cellular extract of mutant A37, a strain that possesses only $IIAB_H^{Man}$ (*5*), there was no stimulation of activity, indicating that $IIAB_H^{Man}$ could not restore mannose PTS activity in mutant G77 on its own (second bar of Figure 6). When the same reaction was carried out with a membrane-free cellular extract of the wild-type strain, which contains both forms of $IIAB^{Man}$, the PEP-dependent phosphorylation activity of 2-DG increased 3- to 4-fold (third bar of Figure 6). This result indicated that the permease portion of the EII^{Man} complex of mutant G77 was still active and that phosphorylation of 2-DG by the mannose:PTS required $IIAB_L^{Man}$. Replacement of the membrane-free cellular extract of the wild-type strain by an extract of a recombinant strain of *E. coli* expressing $IIAB_L^{Man}$ also increased the activity to almost the same level as that observed with a wild-type membrane-free cellular extract (bar 4 of Figure 6). This indicated that $IIAB_L^{Man}$ alone was sufficient to restore mannose:PTS activity in mutant G77. An experiment performed with a cellular extract prepared from a nonrecombinant strain of *E. coli* did not restore mannose:PTS activity in mutant G77 (bar 5, Figure 6), indicating that the stimulation observed with the recombinant strain was caused by $IIAB_L^{Man}$ and not by an *E. coli* protein.

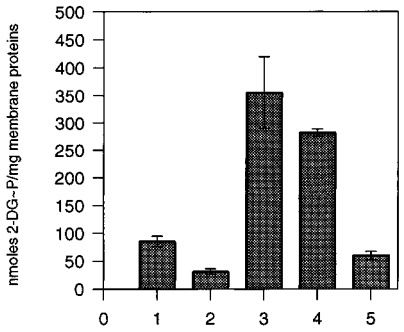

FIGURE 6: 2-DG PTS activities. Reactions were carried out as described in Experimental Procedures using membranes from III^{Man}-deficient mutant G77 as a source of EII^{Man} permease. The activities are expressed as nanomoles of 2-DG~P/μg of membrane protein formed after 2 h at 37 °C. (1) Membranes + cytoplasmic extract from mutant G77; (2) membranes + cytoplasmic extract from III_L^{Man}-defective mutant A37; (3) membranes + cytoplasmic extract from *S. salivarius* ATCC 25975; (4) membranes + cytoplasmic extract from *E. coli* XL-1 Blue expressing III_L^{Man}; (5) membranes + cytoplasmic extract from *E. coli* XL-1 Blue that does not produce III_L^{Man}. The values are the means of two determinations. The bars indicate the standard deviations of the means.

DISCUSSION

The aim of this study was to characterize the PTS proteins III_H^{Man} and III_L^{Man} of *S. salivarius*. These proteins are very difficult to separate. They are coeluted when subjected to different types of chromatography such as ion exchange, gel filtration, hydroxyapatite, and hydrophobic support (*25*). We therefore partially purified them by ion-exchange and gel filtration chromatography and separated them by two-dimensional gel electrophoresis. We were then able to demonstrate that both forms of III^{Man} were phosphorylated on two histidine residues. One phosphate bond was heat-labile (phosphorylation at the N^1 position of the imidazole ring), while the second was heat-resistant (phosphorylation at the N^3 position of the imidazole ring). The presence of two phosphorylation sites on III_H^{Man} and III_L^{Man} was substantiated by circumstantial as well as direct evidence. First, we compared the N-terminal amino acid sequences of these proteins with the N-terminal sequence of IIA domains of the mannose family. The results indicated that (1) the N-terminal sequences of both forms of III^{Man} were identical over the 15 first amino acid residues, (2) the sequences exhibited a high degree of similarity with that of sugar-specific IIA domains of the mannose family (*3*), and (3) both forms of III^{Man} possessed a histidine residue at position 9 that corresponds to the phosphorylation site found in all IIA domains of the mannose EII family (*3*). These results indicated that both forms of III^{Man} possessed a IIA domain

and should therefore both receive their phosphate group from HPr(His∼P). This was actually demonstrated by showing that both proteins were phosphorylated when incubated separately in the presence of purified EI, HPr and [^{32}P]PEP.

Cloning and expression in *E. coli* of the gene coding for III_L^{Man} (unpublished result) allowed us to obtain sufficient amounts of III_L^{Man} to determine the second phosphorylation site of this protein by isolating an internally phosphorylated peptide obtained after exposing purified III_L^{Man} to EI, HPr, and [^{32}P]PEP. The sequence of the phosphorylated peptide indicated the presence of a histidine residue and showed a high degree of similarity with IIB domains of the mannose EII family (*3*). We were unable to isolate a labeled phosphorylated peptide corresponding to the IIA domain inferred from N-terminal amino acid sequence comparison. We do not have any clear explanation for this result.

Taken together, our results show that the proteins we designated III_H^{Man} and III_L^{Man} are two IIAB-type proteins belonging to the mannose class of EIIs. While members of this family possess a IIA domain phosphorylated at the N^3 position of a conserved histidine residue, the IIB domain is phosphorylated at the N^1 position of a histidine residue rather than on a cysteine residue as is the case for the IIB domains of other EII families (*3*). In addition to the amino acid sequence similarities already discussed, the $IIAB^{Man}$ proteins of *S. salivarius* possess structural similarities with the $IIAB^{Man}$ of *E. coli*. Indeed, they all function as homodimers with molecular masses of approximately 70−72 kDa (*26*). The molecular mass of the subunits are 35 kDa for the $IIAB^{Man}$ of *E. coli* (*26*), 35.2 kDa for the $IIAB_L^{Man}$ of *S. salivarius*, and 38.9 kDa for the $IIAB_H^{Man}$ of *S. salivarius* (*4*). Last, both the $IIAB^{Man}$ of *E. coli* and the $IIAB_L^{Man}$ of *S. salivarius* are part of a PTS possessing a broad specificity, the main substrates being mannose, glucose, fructose, and 2-DG (*1, 7*).

Several data suggested that the $IIAB_L^{Man}$ and $IIAB_H^{Man}$ of *S. salivarius* are structurally closely related. First, as already mentioned, both proteins behaved similarly on various chromatographic supports. Second, polyclonal antibodies raised against one form of $IIAB^{Man}$ cross-react, although to a lesser extent, with the other form (*4, 27*). Results obtained in this study also showed that the first 15 N-terminal amino acids are the same in both proteins. These observations suggested that $IIAB_L^{Man}$ may result from a limited proteolysis of $IIAB_H^{Man}$. We have shown, however, that $IIAB_H^{Man}$ and $IIAB_L^{Man}$ had different peptide profiles after protease digestion, indicating that these two proteins are encoded by two different genes. The structural resemblance between these two IIAB proteins and the fact that they are encoded by two genes suggested that one protein is the result of gene duplication. As two forms of $IIAB^{Man}$ have been detected only in *S. salivarius* and *Streptococcus vestibularis*, two members of the salivarius phylogenetic group (*28*), but not in streptococci belonging to other phylogenetic clusters, it appears that the duplication event occurred late in the evolution of streptococci, most probably after the salivarius lineage branched off from the bovis group (*29*). The salivarius cluster is composed of three species: *S. salivarius* and *S. vestibularis*, two oral bacteria, and *Streptococcus thermophilus*, a nonoral bacterium for which the natural ecological niche is unknown (*28, 29*). Surprisingly, *S. thermophilus* is the only member of this phylogenetic cluster that does not possess the high-molecular-mass form of $IIAB^{Man}$ (*27*). This suggests that the environmental conditions that prevail in the oral habitat exert pressure to retain the two forms of $IIAB^{Man}$. On the other hand, it is possible that the gene encoding $IIAB_H^{Man}$ is also present in *S. thermophilus* but that the growth conditions used in our study did not allow its expression.

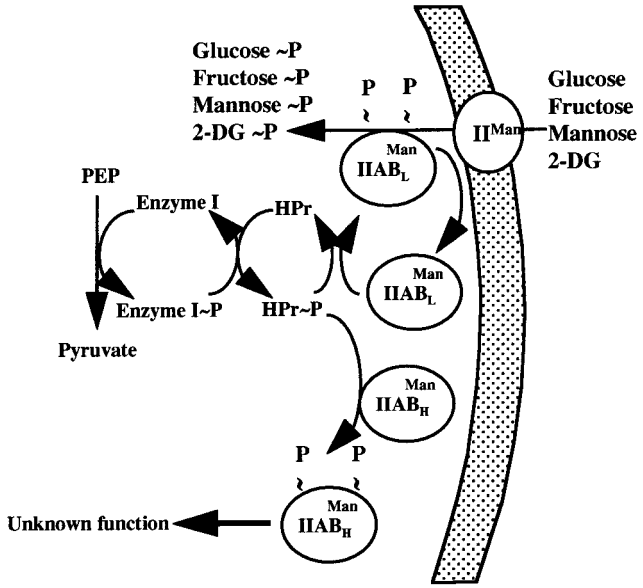

FIGURE 7: Schematic representation of the mannose:PTS of *S. salivarius*. The system can transport and phosphorylate glucose, mannose, fructose, and 2-DG and is composed of the general energy-coupling proteins EI and HPr, a specific uncharacterized membrane-bound module designated II^{Man}, and the dimeric protein $IIAB_L^{Man}$, which possesses two phosphorylation sites per subunit. The function of $IIAB_H^{Man}$ remains unknown. We have demonstrated that phoshotransfer is possible between $IIAB_L^{Man}$ and $IIAB_H^{Man}$. However, the physiological significance of this reaction is unclear.

The finding that the III^{Man} proteins of *S. salivarius* are actually IIAB proteins raises the question of whether both forms of $IIAB^{Man}$ are required for mannose:PTS activity. We have already observed that spontaneous mutants devoid of $IIAB_L^{Man}$ do not grow on mannose and are unable to catalyze the PEP-dependent phosphorylation of mannose and 2-DG, suggesting that the mere presence of $IIAB_H^{Man}$ is not sufficient for mannose-PTS activity (*5, 6*). We later reported the isolation of spontaneous mutants of *S. salivarius* ATCC 25975 that lack both forms of $IIAB^{Man}$ (*6*). These mutants are unable to take up 2-DG and to grow on mannose. In this work, we used one of these mutants (mutant G77) to determine whether the two forms of $IIAB^{Man}$ are involved in the PEP-dependent phosphorylation of 2-DG, a substrate of the mannose:PTS. The results indicated that $IIAB_L^{Man}$, but not $IIAB_H^{Man}$, stimulated the PEP-dependent phosphorylation of 2-DG by membrane of mutant G77, suggesting that $IIAB_H^{Man}$ is not required for mannose:PTS activity. This result suggests that the mannose:PTS of *S. salivarius* is composed of a single, soluble IIAB protein ($IIAB_L^{Man}$) that works in tandem with an uncharacterized membrane module containing the C domain of the II^{Man} complex (Figure 7). This molecular organization is consistent with the finding that only one form of $IIAB^{Man}$ has been detected in several streptococci, in *Lactococcus lactis*, and in *Lactobacillus casei* (*27*). In view of the sequence similarity between $IIAB_H^{Man}$ and $IIAB_L^{Man}$, it is surprising that no cross-functional

reactivity was observed. However, interaction of IIAB proteins with their IIC counterparts involves amino acid sequences in the C-terminal portion of the IIB domain (*26*, *30*). Even though $IIAB_H^{Man}$ and $IIAB_L^{Man}$ display high amino acid sequence homology in the N-terminal region of their IIA domains, the C-terminal sequence of their IIB domains may differ, resulting in molecules with distinct specificity. Nevertheless, we observed that both forms of $IIAB^{Man}$ could transfer their phosphate groups to each other. Although the physiological significance of this cross-talk remains to be elucidated, it suggests that $IIAB_H^{Man}$ is somehow associated with the mannose:PTS. Moreover, the fact that both forms of $IIAB^{Man}$ could transfer their phosphate groups to other unidentified cytoplasmic proteins (Figure 2, lanes 5 and 6) suggests that $IIAB_H^{Man}$ and $IIAB_L^{Man}$ are not only involved in sugar transport via the PTS but may control other physiological processes by phosphorylation.

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