JOURNAL OF BACTERIOLOGY, Sept. 1976, p. 1298-1306
Vol. 127, No. 3

Printed in U.S.A.

Mycoplasma Phosphoenolpyruvate-Dependent Sugar Phosphotransferase System: Purification and Characterization of the Phosphocarrier Protein

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Received for publication 28 May 1976

The *Mycoplasma* phosphoenolpyruvate-dependent sugar phosphotransferase system consists of three components: a membrane-bound enzyme II, a soluble enzyme I, and a soluble phosphocarrier protein, HPr. The HPr has been purified to homogeneity by a combination of ammonium sulfate precipitations, gel filtration, and diethylaminoethyl, carboxymethyl Bio-Gel A, and hydroxylapatite column chromatography. The purified protein is relatively heat stable (ca. 50% activity survives 30 min of boiling) and has a molecular weight of ca. 10,000 (determined by sodium dodecyl sulfate-gel electrophoresis and amino acid analysis). It contains a single histidine residue per molecule and can be totally inactivated by photooxidation with Rose Bengal dye. Although the mycoplasma HPr is very similar to that of *Escherichia coli*, it shows no significant association with antiserum produced against *E. coli* HPr.

The occurrence of a phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) in *Mycoplasma* was reported by Van Demark and Plackett (15) and Cirillo and Razin (2). In subsequent preliminary reports (V. P. Cirillo, 1973, Abstr. First Int. Congr. Bacteriol., Jerusalem, Israel, vol. I, p. 57, and V. P. Cirillo, Abstr. Annu. Meet. Am. Soc. Microbiol., 1973, p. 126), the PTS of *Mycoplasma capricolum* was shown to be similar to that of *Escherichia coli* (1, 7, 8, 9, 10, 12, 13) in consisting of three components: (i) a membrane-bound enzyme, enzyme II; (ii) a low-molecular-weight (ca. 10,000), heat-stable phosphocarrier protein, HPr; and (iii) a high-molecular-weight (ca. 150,000) soluble enzyme, enzyme I. Complementation among the components of the *Mycoplasma* PTS and that of *E. coli* showed that while the *Mycoplasma* P ~ HPr is fully active as a phosphocarrier with *E. coli* enzyme II, the *E. coli* P ~ HPr is poorly active with *Mycoplasma* enzyme II. The nonreciprocal activity of the phosphocarrier proteins between the two systems affords a unique opportunity to understand the structural requirements for phosphocarrier activity and to compare the structures of the phosphocarrier protein of a "primitive," small genome mycoplasma (16) with that of a "higher" bacterium. The following report describes the purification and partial characterization of a *Mycoplasma* HPr.

MATERIALS AND METHODS

Organisms and growth conditions. *M. capricolum* (the Kid strain) was obtained from Joseph Tully of the National Institutes of Health. The organisms were grown in modified Edwards medium at pH 8.0 containing 0.042 M NaCl, 0.014 M K_2HPO_4, 0.52% (wt/vol) glucose, 0.5% (wt/vol) peptone, 0.7% (wt/vol) yeast extract, 1.3% (wt/vol) heart infusion broth, 20 ml of bovine serum fraction A (GIBCO, Grand Island, N.Y.) per liter, 28.0 mg of deoxyribonucleic acid per liter, and 1.0 million units of penicillin G per liter. The cells were grown in stationary 2-liter volume Erlenmeyer flasks containing 500 ml of liquid medium at 37°C. The cells were grown up to the mid-exponential phase of growth, by which time the medium pH had fallen to between pH 5.5 and pH 6.0. The culture was chilled to 0°C, and the cells were harvested by using a Sorvall RC 2-B centrifuge and an HS-4 rotor at 9,000 × *g* for 10 min. The average cell yield ranged from 0.7 to 1.0 g of wet cells per liter of medium. The harvested cells were washed twice with ice-cold buffer containing 0.25 M NaCl and either used directly for fractionation of PTS components or stored at −20°C. All further steps were carried out at 0 to 4°C. *E. coli* K-12 was grown in modified Edwards medium without penicillin G or bovine serum. All other conditions were the same.

Preparation of crude extracts. Washed cells were suspended thoroughly in β-buffer [150 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM β-mercaptoethanol, and 0.1 mM dithiothreitol (DTT)]. A typical preparation contained 4 to 5 g of wet cells in 15 to 20 ml of β-

buffer. The cell suspensions were then subjected to ultrasonic oscillation in a sonifier (Branson model 6125) at the power output of 5 for three successive 30-s periods. The viscous, sonically treated cell suspensions were treated with 0.1 to 0.2 mg of dexyribonuclease per ml, and the unbroken cells and cellular debris were separated from the extracts by centrifugation at 15,000 × *g* for 20 min in a Sorvall SS-34 rotor.

Separation of membranes and soluble fraction. The crude extracts were further centrifuged at 101,962 × *g* for 90 min at 4°C in a Beckman model L-5-65 ultracentrifuge in a 65 rotor. The supernatant solution was carefully separated and saved for fractionation of enzyme I and HPr. The isolated membranes were thoroughly resuspended with the aid of a small Bellco glass homogenizer in β-buffer and recentrifuged at 159,000 × *g* for 90 min at 4°C. After this second centrifugation, the translucent membrane fraction was resuspended in β-buffer and either used immediately or stored at −20°C. The supernatant fraction from this second centrifugation was added to the previously collected supernatant fraction. The washed membrane material was the source of enzyme II in all PTS assays described below.

Purification of HPr. The supernatant fraction obtained by centrifugation at 101,962 to 159,000 × *g* was used for purification of HPr. All steps of fractionation were carried out at 0 to 4°C. The steps of purification described below are listed in Table 1, with the indicated specific activities and purifications achieved for each step.

Step 1: Crude supernatant. The crude supernatant is the supernatant material obtained from the two high-speed centrifugations described above.

Step 2: Ammonium sulfate precipitation. The crude soluble fraction from step 1 was adjusted to 100% saturation of $(NH_4)_2SO_4$ by addition of crystalline enzyme-grade ammonium sulfate (Schwarz/Mann, Orangeburg, N.Y.) over a period of 30 to 45 min with constant stirring. The apparent pH of the mixture measured with a glass electrode was maintained at 7.4, and the saturated solution was stirred for 12 h at 4°C. The precipitated total soluble proteins were removed by centrifugation at 30,000 × *g* for 30 min and resuspended in a minimal volume of β-buffer.

Step 3: Bio-Gel P-10 gel chromatography. The ammonium sulfate-precipitated protein fraction from step 2 was applied to a Bio-Gel P-10 column (1.5 by 30.0 cm) preequilibrated with β-buffer. The column was eluted with this same buffer at a flow rate of about 0.6 ml/min. The column was precalibrated with blue dextran 2,000 and cytochrome *c*. The Bio-Gel P-10 chromatography effectively separated almost all the enzyme I from the HPr; enzyme I eluted in the void volume whereas HPr was eluted slightly after the cytochrome *c*. The enzyme I activity from the void volume from Bio-Gel P-10 columns was the source of enzyme I in all the assays described below.

Step 4: Ammonium sulfate fractionation. The active fraction from step 3 was adjusted to 65% saturation by adding crystalline, enzyme-grade ammonium sulfate over a period of 30 min to 1.0 h with continuous stirring. During such addition, the apparent pH was maintained at 7.4 by the addition of ice-cold NH_4OH. Afer 2 to 4 h of stirring, the precipitate was removed by centrifugation at 30,000 × *g* for 30 min and discarded. Excess ammonium sulfate was added, as described above, until 100% saturation was achieved and the solution was stirred for an additional 12 h at 4°C. The precipitated proteins were removed by a brief centrifugation at 30,000 × *g*, resuspended in a minimal amount of phosphate buffer, and dialyzed against phosphate buffer (diethylaminoethyl [DEAE] Bio-Gel A buffer; see step 5 below) for a period of 24 h to remove the ammonium sulfate.

Step 5: DEAE Bio-Gel A chromatography. The dialyzed active fraction from Step 4 was applied to DEAE Bio-Gel A columns (1.5 by 12.5 cm) previously equilibrated with phosphate buffer containing 10 mM potassium phosphate, pH 7.4, 0.1 mM DTT, and 0.1 mM EDTA. The column was eluted with a 200-ml portion of phosphate buffer containing the following increasing concentrations of KCl: 0, 0.05, 0.10, 0.15, and 0.2 M. Three fractions with HPr activity were detected. That with the highest was eluted with the KCl-free wash; the other two, with lower specific activity, were eluted by 0.05 M and 0.1 M KCl (Fig. 1A).

Step 6: CM Bio-Gel A chromatography. The active fractions from the KCl-free wash of the DEAE Bio-Gel A column (fraction I) were pooled and dialyzed against sodium acetate buffer (10 mM Na acetate, pH 4.9, 0.1 mM DTT, and 0.1 mM EDTA) for 24 h with three changes of buffer and applied to a carboxymethyl (CM) Bio-Gel A column (1.5 by 12.5 cm) previously equilibrated with sodium acetate buffer. The column was eluted with a linear gradient at 0 to 0.3 M NaCl in sodium acetate buffer (300 ml total). HPr activity was eluted as a single component at about 0.1 M NaCl, which was coincident with a single protein component.

Step 7: Hydroxylapatite column. The fractions showing HPr activity from the CM Bio-Gel A column were pooled, dialyzed against potassium phosphate buffer (1 mM potassium phosphate, pH 7.0, 0.1 mM DTT, 0.1 mM EDTA) for 24 h with four changes of buffer, and applied to a hydroxylapatite column, Bio-Gel HTP (1.5 by 10 cm). The column was preequilibrated with 1 mM potassium phosphate buffer, pH 7.0, and eluted with a linear gradient of potassium phosphate buffer, pH 7.0, from 1 to 300 mM (300 ml total). The HPr activity was eluted in a single peak at approximately 70 mM potassium phosphate. The HPr activity was coincident with a single protein fraction (Fig. 1C), and the specific activity of each fraction was identical.

Enzyme assays. The HPr activity was measured by an assay system which involves the phosphorylation of α-methyl-D-glucopyranoside (α-MG) in the presence of fixed amounts of enzyme I and II. The assay medium, essentially a modification of Kundig and Roseman enzyme assay A (10), contained the following components in a final volume of 250 μl, at pH 7.4: $MgCl_2$, 2 μmol; K_2HPO_4, 20 μmol; KF, 4.0 μmol; phosphoenolpyruvate-tricyclohexylamine salt, 4.0 μmol; DTT, 0.5 μmol; and spermine

tetrachloride, 0.5 μmol; 350 to 750 μg of protein of enzyme II and 350 to 500 μg of protein of enzyme I. The assay was initiated at 37°C by addition of 0.25 μmol of ^{14}C-labeled α-MG (0.5 μCi/μmol) and terminated, usually after 60 min, by heating in a boiling-water bath for 4 min. Control incubations contained separate components (i.e., enzyme II or enzyme I) or lacked HPr. The phosphorylated α-MG was separated from the unphosphorylated sugar by passing through a Bio-Rad AG 1-X2 (50- to 100-mesh) anion-exchange resin column (2). The unphosphorylated α-MG was washed from the column by addition of 20 ml of deionized water, and the phosphorylated sugar was eluted with a 6.0-ml portion of 1.0 M LiCl solution, The eluate, collected directly in a scintillation counting vial, was counted in a scintillation spectrometer (Intertechnique model SL-30), after the addition of 15.0 ml of scintillation fluid consisting of 666 ml of scintillation-grade toluene, 333 ml of Triton X-100, 5.5 g of 2,5-diphenyloxazole, and 125 mg of dimethyl-1,4-bis-2-(5-phenyloxazole)benzene (10).

The HPr and enzyme I fractions are not significantly contaminated with each other or enzyme II; however, enzyme II preparations (i.e., washed membranes) are constantly contaminated with low levels of enzyme I and HPr. Specific activity is expressed as nanomoles of α-MG phosphorylated per milligram of HPr protein per minute at 37°C. Since HPr is not an enzyme but a substrate of both enzyme I (before phosphorylation) and enzyme II (after phosphorylation), "units of activity" for a given amount of HPr depends upon the amount and activity of enzyme I and II. Because these were varied in different experiments, and different batches of enzyme I and enzyme II had somewhat different activities, comparisons of specific activity for a given purification regimen of HPr required that material from all purification steps be assayed with the same amounts and batch of enzyme I and enzyme II (see reference 14 on this same point).

Analytical techniques. The protein concentration of soluble enzyme and membrane fractions was determined by the method of Lowry et al. (11) using bovine serum albumin as standard.

Electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels at 7.5% monomer concentration was performed by the method of Fairbanks et al. (3). The electrophoresis buffer used in both upper and lower chambers contained 1% SDS. The samples, 1 to 10 μg of purified HPr protein, were preincubated with a solubilizer containing 1% SDS, 5 to 10% sucrose, 10 mM Tris-hydrochloride, pH 8.0, 1.0 mM EDTA, 40 mM DTT, and 10 μg of pyronin-Y dye per ml, for 30 min at 37°C. Electrophoresis was carried out at room temperature in a gel electrophoresis chamber (Bio-Rad model 150) with a current of 4.0 mA per tube supplied by a Gelman power supply unit (model 38206). The running time under these conditions was about 3.0 h. The gels were stained for protein with Coomassie brilliant blue and destained in 10% acetic acid in a diffusion destainer (Bio-Rad model 170).

Disc electrophoresis in non-SDS, pH 8.7, polyacrylamide gels at 5.0% monomer concentration was also performed. The separating gels were polymerized in a solution mixture containing 1 part solution A, 0.87 part solution C, 4 parts 0.15% ammonium persulfate, 2.11 parts deionized water, and tetramethylethylenediamine (TEMED) at 0.025% final concentration. Solution A contains 48 ml of 1.0 M HCl, 36.3 g of Tris-base, and water to 100 ml, pH 8.9. Solution C contains 45.0 g of acrylamide, 1.2 g of methylene bisacrylamide, and water to 100 ml. Stacking gels were polymerized in a solution containing 1 part solution B, 1 part solution D, 1 part water, 1 part 0.15% ammonium persulfate, and TEMED to a final concentration of 0.0025%. Solution B contains 6.4 ml of 1.0 M H_3PO_4, 1.425 g of Tris-base, and water to 100 ml, pH 6.9. Solution D contains 10.0 g of acrylamide, 2.5 g of methylene bisacrylamide, and water to 100 ml. The sample in 100 μl of buffer contained 10% sucrose, 0.01 M β-mercaptoethanol, 1/5 diluted solution B, and 1 μl of 0.05% bromophenol blue dye. Electrophoresis was performed at 4°C with a current of 2.5 mA per tube. The time for indicator dye bromophenol blue to move 6.0 cm was about 2.0 h. The gels were stained for protein with Coomassie blue and destained in 10% acetic acid.

Amino acid composition. Amino acid analyses were carried out with an automatic amino acid analyzer (JEOL-JLC-6AH). For this purpose, 20 to 30 μg of HPr was lyophilized. In order to estimate the amide content, the following treatment was carried out: a 0.5-ml portion of 0.2 N borate buffer, pH 9.0, was added to the lyophilized HPr, boiled for a period of 10 min, and dried in a desiccator over concentrated H_2SO_4 as desiccant (6). Hydrolysis of the HPr was then carried out in a sealed evacuated tube in 0.4 ml of constant boiling HCl for a period of either 24 or 72 h at 110°C. A performic acid oxidation was carried out to determine cysteine content according to the method of Hirs (5). Tryptophan content was determined by the method of Goodwin and Morton (4).

Photooxidation of HPr. Photooxidation of HPr was carried out by the following modification of the procedure of Westhead (9, 17). Inactivation was accomplished by exposing a solution containing 0.3 mg of HPr (step 4) and 15 μg of Rose Bengal dye (in 1.0 ml of 0.05 M Tris-hydrochloride buffer, pH 8.0, containing 0.01 mM EDTA) to the light of an 800-W slide projector bulb for a period of 45 min at 25°C. The light source was kept 10 cm away from the sample. The entire solution was then passed through a Bio-Rad column (0.7 by 4 cm) of AG 1-X2 anion-exchange resin in the chloride form (200 to 400 mesh) to remove the acidic dye. The HPr came through the column, while the dye stayed in the resin. A 50-μl fraction of the column eluate was immediately assayed for HPr activity.

Anti-*E. coli* antiserum-HPr interactions. Antiserum and *E. coli* or *Mycoplasma* HPr were mixed in equal portions (0.35 ml) and incubated at 4°C for 2 h. After incubation, 0.5 ml of the mixture was passed through a Bio-Gel P-100 column (8 mm by 18 cm) and eluted with β-buffer. Fractions (1.4 ml) were collected, and 50-μl portions of each fraction were assayed for HPr activity as described above.

Source of materials. Uniformly ^{14}C-labeled α-methyl-D-glucopyranoside was purchased from Amersham-Searle, England. DTT and phosphoenolpyruvate-tricyclohexamine salt were purchase from the Sigma Chemical Co., St. Louis, Mo.; bovine serum fraction A (1X) from the Grand Island Biological Co., Grand Island, N.Y. Anion-exchange resin AG 1-X2 (50 to 100 mesh), Bio-Gel P-10, and P-30 polyacrylamide beads, anion exchanger DEAE Bio-Gel A, cation exchanger CM Bio-Gel A, hydroxylapatite Bio-Gel HTP, and materials for polyacrylamide disc gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, Calif. All other chemicals were bought from available commercial sources and were of analytical or reagent grade. Purified *E. coli* HPr and goat anti-*E. coli* HPr-antiserum were kindly provided by S. Roseman of the Johns Hopkins University, Baltimore, Md.

RESULTS

Purification. A scheme similar to those used for the purification of the *E. coli* (1) and *Staphylococcus aureus* (14) HPr was adapted to purify the *Mycoplasma* protein by ammonium sulfate precipitations, gel filtration, and column chromatography, with the results shown in Fig. 1 and Table 1.

The proteins of the step 1 crude supernatant fraction obtained from the pooled, high-speed centrifugation of extracts were concentrated by precipitation with saturated ammonium sulfate (step 2) followed by gel filtration (step 3). (These two steps removed an inhibitor which accounts for the increase in activity shown in Table 1 between steps 1 and 3.) Gel filtration of the concentrated material (step 3) quantitatively separated the enzyme I from HPr; enzyme I was eluted in the void volume and HPr in the fraction corresponding to 10,000 molecular weight.

After ammonium sulfate fractionation and dialysis of the precipitate formed between 65 and 100% saturation (step 4), the HPr was applied to a DEAE column. Three fractions were obtained (step 5 and Fig. 1A). The major fraction (fraction I), accounting for about 90% of the total activity, did not stick to the DEAE column and came through in the phosphate buffer wash. The two remaining fractions, II and III, were eluted by 0.05 M and 0.1 M KCl, respectively. Only the high-specific-activity fraction I (which eluted between the arrows of Fig. 1A) was used for subsequent purification on CM Bio-Gel A and hydroxylapatite (HA) columns. The CM Bio-Gel A column was eluted with a linear NaCl gradient from 0 to 0.3 M (step 6, Fig. 1B); the HA column was eluted with a linear phosphate gradient from 0.001 to 0.3 M (step 7, Fig. 1C). The final product eluted from the HA column was shown to be homogeneous by electrophoresis in SDS and in the pH 8.7 non-SDS gel (Fig. 4A).

A comparison of the migration in an SDS gel of the purified HPr with several molecular-

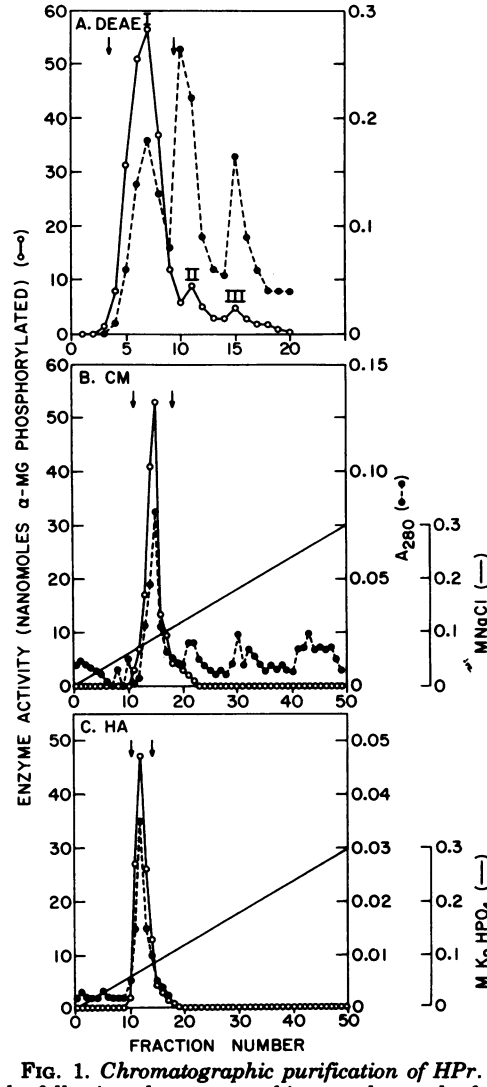

FIG. 1. *Chromatographic purification of HPr. In the following chromatographic procedures, the fractions were uniformly of 5.0 ml. (A) The DEAE Bio-Gel A column was eluted successively with phosphate buffer alone (fraction I), buffer plus 0.05 M KCl (fraction II), and buffer plus 0.1 M KCl (fraction III). Only fraction I between the arrows was used for the carboxymethyl (CM) column. (B) The CM Bio-Gel A column was eluted with a linear gradient of NaCl from 0 to 0.3 M. Only fractions between the arrows were used for the hydroxylapatite (HA) column. (C) The HA Bio-Gel HTP column was eluted with a linear phosphate buffer gradient from 0.001 to 0.3 M phosphate buffer. The fractions between the arrows were used for characterization of purified HPr.*

TABLE 1. *Purification of HPr*

Fractionation step	Vol (ml)	Total protein (mg)	Total activity (units)[a]	Relative sp act[b]	Fold purification
1. Crude supernatant	25.0	400	308	0.77	1
2. Ammonium sulfate: 100% saturation	5.0	395	ND	ND	ND
3. Bio-Gel P-10 gel filtration	113.5	25.5	1,626	63.77	82
4. Ammonium sulfate: 65–100% saturation	15.75	3.1	524	169.0	219
5. DEAE Bio-Gel A					
Fraction I	35.0	1.22	365	299.34	388
Fraction II	20.0	0.88	37	42.44	
Fraction III	15.0	0.51	16	31.87	
6. CM Bio-Gel A[c]	40.0	0.616	253	421.8	548
7. Hydroxylapatite Bio-Gel HTP	20.0	0.302	199	664.0	862

[a] Nanomoles of α-MG phosphorylated per minute at 37°C. ND, Not done.
[b] Nanomoles of α-MG phosphorylated per milligram of HPr protein per minute at 37°C.
[c] CM, Carboxymethyl.

weight standards (i.e., insulin, bungarotoxin, and cytochrome *c*) suggests a molecular weight of ca. 10,000, conforming with the molecular weight estimated from the elution from gel filtration columns when using the same standards.

The data of Fig. 2 show that, under the assay conditions used in this study, PTS activity depends linearly on the amount of purified HPr (Fig. 2A) and is constant over a 2-h period for a given HPr concentration (Fig. 2B).

Characterization. The heat stability of purified HPr is shown in Fig. 3. Heating at 60 to 100°C leads to a rapid inactivation of 40 to 60% of the initial activity, respectively, with no further loss of activity up to 30 min of heating at either temperature.

As previously mentioned, the final product of purification was shown to be homogeneous by electrophoresis in SDS and in a pH 8.7 non-SDS gel (Fig. 4A). For comparison, purified *E. coli* HPr (kindly provided by Saul Roseman) was also subjected to electrophoresis in these two gel systems alone (4B) or in a mixture with the *Mycoplasma* HPr (4C). While the two proteins have different mobilities in the alkaline non-SDS gel, they comigrate in SDS. From the mobility in the SDS gels in comparison with molecular-weight standards (i.e., insulin [5,800], bungarotoxin [8,000] and cytochrome *c* [12,700]), the molecular weights of the *E. coli* and *Mycoplasma* proteins are estimated to be ca. 10,000. Since this is the same molecular weight estimated by elution from gel filtration columns (step 3), the HPr protein can be said to be a monomer.

The ability of the PTS components of *Mycoplasma* and *E. coli* to complement with each other was studied using membranes and soluble fractions of each organism. The results shown in Table 2 indicate that, whereas *Mycoplasma* P ~ HPr (formed by the soluble fraction) can function equally well with *E. coli* or *Mycoplasma* enzyme II, the *E. coli* P ~ HPr functions only 10% as well with *Mycoplasma* enzyme II as with *E. coli* enzyme II.

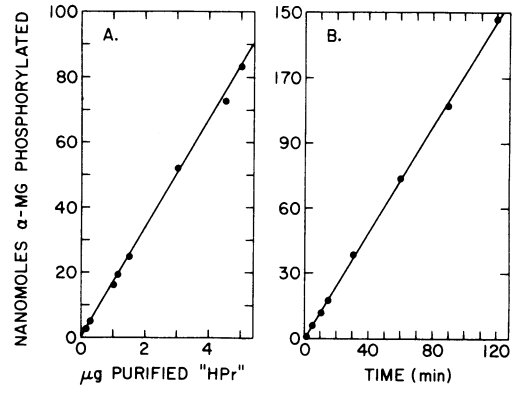

FIG. 2. *(A) Dependence of phosphotransferase activity on HPr concentration. The assays were carried out in a final volume of 0.25 ml; HPr varied from 0.1 to 5.0 μg of protein; enzyme I, 0.4 mg of protein; enzyme II, 0.75 mg of protein. Incubation time, 60 min. (B) Time dependence. HPr, 3.0 μg of protein; enzyme I, 0.4 mg of protein; enzyme II, 1.0 mg of protein. Time of incubation varied from 0 to 120 min. The final volume was kept at 0.25 ml.*

Further dissection of the *Mycoplasma* and *E. coli* PTS involved complementation using isolated enzyme I, enzyme II, and HPr. The first four experiments of Table 3 are equivalent to the complementations reported in Table 2 using a combination of the isolated components (enzyme I and HPr) instead of the crude soluble fraction. The results are identical to those ob-

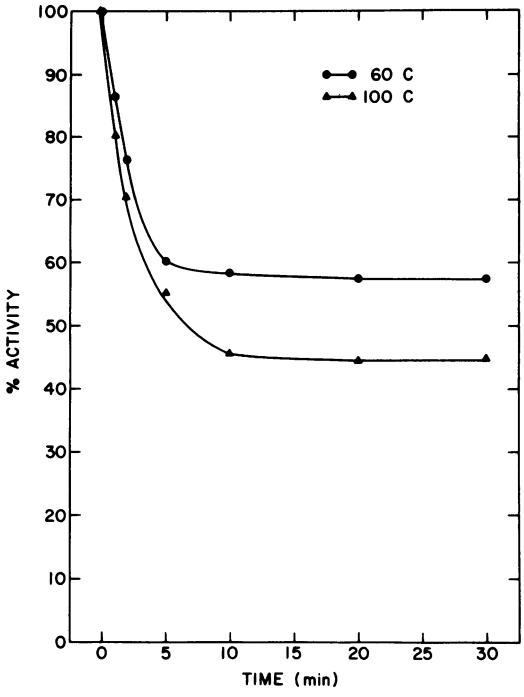

FIG. 3. *HPr heat stability. PTS activity was determined by using 1.0 μg of HPr protein previously heated for the times indicated either at 60 or 100°C. The assay employed 0.5 mg of enzyme I protein, 1.0 mg of enzyme II protein; incubation time, 60 min. Results expressed as percentage of unheated HPr control. A 100% activity represents 4.67 nmol of α-MG phosphorylated per 60 min.*

tained with the crude extracts. Experiments 5 through 8 are heterologous combinations of *Mycoplasma* and *E. coli* enzyme I and HPr. Experiments 5 and 6 are a measure of the ability of *E. coli* enzyme I to phosphorylate *Mycoplasma* HPr. Using *E. coli* membranes, the phosphorylation ability is 34%; using *Mycoplasma* membranes, it is 17%. Experiments 7 and 8 are a measure of the ability of *Mycoplasma* enzyme I to phosphorylate *E. coli* HPr. A comparison of the results of experiments 7 and 8 confirms those of experiment 2 that *E. coli* P ~ HPr is a poor substrate for *Mycoplasma* enzyme II. The data of experiment 8 show that *Mycoplasma* enzyme I phosphorylates *E. coli* HPr only ca. 20% as well as it does the *Mycoplasma* HPr (experiment 3).

The ability of *Mycoplasma* HPr to serve as a phosphocarrier between *Mycoplasma* enzyme I and *E. coli* enzyme II suggests significant homology. To determine whether this enzymological homology extends to the serological level, interaction between *Mycoplasma* HPr and antiserum against the *E. coli* HPr was measured. Because the antiserum-HPr interaction does not produce a precipitation, the antiserum-HPr interaction was measured by elution of the complex from a Bio-Gel P-100 column. In such a column the large-molecular-weight antibodies and the antiserum-HPr complexes pass through in the void volume, whereas the HPr is retained, eluting in the 10,000-molecular-weight fraction. The elution data of Fig. 5 show that under conditions in which more than 50% of the *E. coli* HPr combined with the antiserum, no detectable *Mycoplasma* HPr-antiserum in-

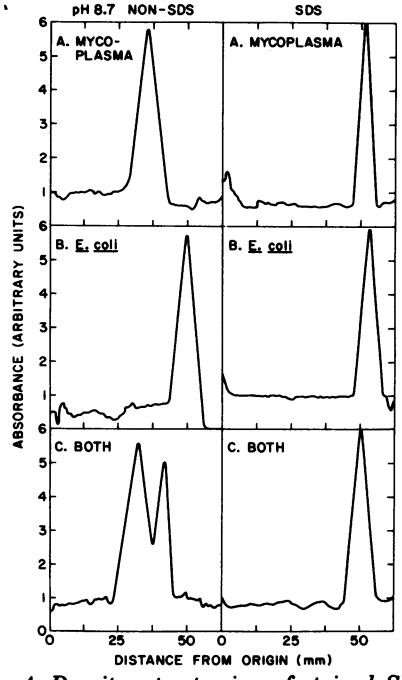

FIG. 4. *Densitometer tracing of stained SDS or non-SDS, pH 8.7, gels. (A) Mycoplasma HPr alone; (B) E. coli HPr alone; (C) Mycoplasma + E. coli HPr mixture.*

TABLE 2. *In vitro complementation between Mycoplasma and E. coli phosphotransferase system*[a]

Expt no.	Source of membrane fraction (enzyme II)	Source of soluble fraction (enzyme I and HPr)	α-MG phosphorylated/min at 37°C (nmol)
1	*Mycoplasma*	*Mycoplasma*	0.97
2	*Mycoplasma*	*E. coli*	0.1
3	*E. coli*	*Mycoplasma*	0.8
4	*E. coli*	*E. coli*	0.78

[a] The assays were carried out in a final volume of 0.25 ml. In all cases, the reaction mixture contained 1.0 mg of membrane protein and 0.75 mg of soluble protein. The reaction mixtures were incubated for 60 min at 37°C.

TABLE 3. *In vitro complementation among isolated components of the phosphotransferase system (enzyme II, enzyme I, and HPr) of Mycoplasma and E. coli*[a]

Expt no.	Source of enzyme II	Source of enzyme I	Source of HPr	α-MG phosphorylated/min at 37°C (nmol)
1	*Mycoplasma*	*Mycoplasma*	*Mycoplasma*	0.69
2	*Mycoplasma*	*E. coli*	*E. coli*	0.06
3	*E. coli*	*Mycoplasma*	*Mycoplasma*	0.44
4	*E. coli*	*E. coli*	*E. coli*	0.43
5	*Mycoplasma*	*E. coli*	*Mycoplasma*	0.12
6	*E. coli*	*E. coli*	*Mycoplasma*	0.15
7	*Mycoplasma*	*Mycoplasma*	*E. coli*	0.015
8	*E. coli*	*Mycoplasma*	*E. coli*	0.09

[a] The assay conditions are described in the text. In all cases, the reaction mixture contained 0.65 mg of membrane protein (source of enzyme II), 1.0 mg of enzyme I, and 0.15 mg of HPr. The source of enzyme I and HPr was Bio-Gel P-10 column eluates (step 3).

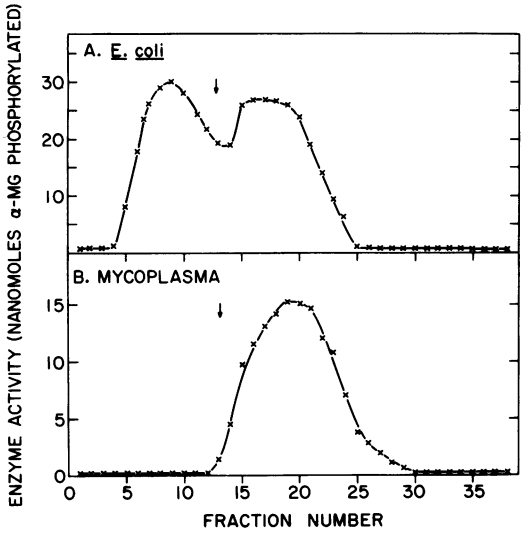

FIG. 5. *Anti-E. coli-antiserum interaction with E. coli versus Mycoplasma HPr. HPr fractions from Mycoplasma capricolum and E. coli (strain K-12) purified to the equivalence of step 3 of Table 1 were mixed in equal portions (0.35 ml) with anti-E. coli HPr goat antiserum and incubated for 2 h at 4°C. After the incubation, a 0.5-ml portion of the mixture was applied to a Bio-Gel P-100 column (8 mm by 18 cm) and eluted with β-buffer into 1.4-ml fractions; 50-μl portions of each fraction were assayed for HPr activity. The arrows in the figure mark the limit of the void volume of the column.*

teraction occurred. There was no evidence that the antiserum was inhibitory to either *E. coli* or *Mycoplasma* HPr activity since all the activity added to the columns was recovered from the columns either as a single component for *Mycoplasma* or two components for *E. coli*.

As a final characterization, the amino acid composition of the *Mycoplasma* HPr was determined (Table 4). For comparison, the amino acid compositions of the *E. coli* (1) and *S. aureus* (14) proteins are also shown.

TABLE 4. *Amino acid analysis of HPr from Mycoplasma compared with that of Staphylococcus aureus and E. coli*[a]

Amino acid	Organism		
	Mycoplasma	*S. aureus*	*E. coli*
Lysine	9.6[b] ± 0.5	9.4[c]	8.1[d]
Histidine	1.0 ± 0.2	1.02	2.0
Arginine	1.1 − 0.2	1.04	0.96
Aspartic acid	10.5 ± 0.7	9.4	3.6
Threonine	5.9 ± 0.6	4.6	10.0
Serine	5.6 ± 0.6	6.2	6.9
Glutamic acid	10.1 ± 0.8	8.3	13.5
Proline	2.4 ± 0.3	1.2	2.2
Glycine	7.8 ± 0.8	6.0	6.7
Alanine	10.7 ± 0.8	5.7	9.4
Valine	3.7 ± 0.5	4.2	7.1
Methionine	2.9 ± 0.2	3.2	2.1
Isoleucine	8.4 ± 0.6	6.8	3.4
Leucine	5.7 ± 0.8	5.1	8.5
Tyrosine	0	2.2	0
Phenylalanine	2.8 ± 0.6	0.94	4.0
Cysteine[e]	1.7	0	0
Tryptophan[f]	0	0	0
Ammonia	7.9		9.2
Molecular weight (from summation)	9,506	8,630	9,537

[a] Reproduced from Anderson et al. (1) and Simoni et al. (14) with kind permission. The values represent average of five independent determinations. The values given are not corrected for loss during hydrolysis. Experimental procedures for amino acid analysis are given in the text.

[b] Residues per 1.0 mol of histidine; the protein was hydrolyzed for 24 h.

[c] Residues per 6.0 mol of glycine; the protein was hydrolyzed for 20 h (14).

[d] Residues per 2.0 mol of histidine; the protein was hydrolyzed for 24 h (1).

[e] Determined as cysteic acid (5).

[f] Determined spectrophotometrically (4).

Although the role of histidine as the phosphoryl carrier of HPr has not been tested directly, it was found that exposure of HPr to

photooxidation conditions which specifically destroy histidine residues, as described by Westhead (17), completely inactivates *Mycoplasma* HPr. Exposure of HPr to the Rose Bengal dye in the absence of light or exposure of HPr to light in the absence of the dye causes no loss of activity. (The data are not shown.)

DISCUSSION

The *Mycoplasma* PTS is comparable to that of *E. coli* in consisting of a membrane-bound enzyme II, two soluble proteins, a higher-molecular-weight enzyme I, and a low-molecular-weight phosphocarrier protein. Significant homology between the two systems was demonstrated by the fact that the soluble components of the *Mycoplasma* system (containing enzyme I and HPr) could replace the soluble components of *E. coli* for PTS activity with *E. coli* enzyme II. Complementation experiments with the isolated components of both *Mycoplasma* and *E. coli* have shown that the *Mycoplasma* HPr functions nearly as well as the *E. coli* HPr itself with *E. coli* enzyme II. The *Mycoplasma* HPr is also similar to the *E. coli* protein with respect to its molecular weight, heat stability, chromatographic characteristics, and photoinactivation in the presence of Rose Bengal dye. The elution of the *Mycoplasma* HPr from DEAE columns shows striking similarities with that of *E. coli* HPr (1). Three fractions are isolated from DEAE columns at pH 7.4: a major cationic protein (which does not stick to the DEAE column) and two minor anionic protein fractions which are eluted by increasing concentrations of KCl in phosphate buffer. In *E. coli*, the identical elution pattern is observed and has been attributed to the loss of amide groups from asparagine and glutamine residues during purification, producing successively more anionic proteins. The high ammonia and aspartic and glutamic acid concentrations found in the amino acid analysis of proteins may have a similar origin. In the case of *Mycoplasma*, however, the major cationic protein is over 90% of the total HPr activity, whereas in *E. coli* it constitutes only about 60% of the total (1).

In spite of the many similarities between the *Mycoplasma* and *E. coli* proteins, significant differences also exist. Thus, although the *Mycoplasma* P ~ HPr can function as a phosphoryl carrier with *E. coli* enzyme II, *E. coli* P ~ HPr functions only poorly with *Mycoplasma* enzyme II. The amino acid analysis also reveals a number of important differences from that of the *E. coli* protein, the most important being the occurrence of only a single histidine residue per molecule. In this respect the *Mycoplasma* protein resembles that of *S. aureus*, which also contains only one histidine residue (14). In addition, the *Mycoplasma* protein contains cysteine, which is absent from both the *E. coli* and *S. aureus* proteins. Finally, there seems to be no significant immunological cross-reactivity between the two proteins judged from the inability of antiserum to the *E. coli* HPr to complex with the *Mycoplasma* HPr.

Phosphorylation of the *Mycoplasma* HPr has not been demonstrated directly. However, its ability to serve as a phosphocarrier between *E. coli* enzyme I and enzyme II and its photoinactivation under conditions in which histidine residues undergo photooxidation (9, 17) strongly suggest that it is phosphorylated at a histidine residue.

Extension of complementation studies between the components of the PTS of *M. capricolum* and those of other mycoplasmas and other bacteria are continuing and are expected to provide significant information on the possible "kinship" between mycoplasmas and other bacteria. Preliminary, unpublished data have already shown that the PTS components from all the mycoplasmas studied so far (*Mycoplasma* and *Spiroplasma* species) completely complement each other, whereas the PTS components of *M. capricolum* show nonreciprocal complementation with those of *Arthrobacter pyridinolis*.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI 11506 from the National Institute of Allergy and Infectious Diseases.

We gratefully acknowledge the advice of Masayori Inouye on the amino acid analysis and thank Sumiko Inouye for the determinations of the amino acid composition. We are also grateful to Saul Roseman and Werner Kundig for samples of *E. coli* HPr and the anti-HPr antiserum and to William Steinfeld for his dedicated assistance.

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