JOURNAL OF BACTERIOLOGY, Sept. 1977, p. 988-996
Vol. 131, No. 3

Printed in U.S.A

Mycoplasma Phosphoenolpyruvate-Dependent Sugar Phosphotransferase System: Purification and Characterization of Enzyme I

ABUL H. JAFFOR ULLAH AND VINCENT P. CIRILLO*

Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

Received for publication 14 April 1977

The *Mycoplasma* phosphoenolpyruvate-dependent sugar phosphotransferase system consists of three components: a membrane-bound enzyme II, a soluble phosphocarrier protein (HPr), and a soluble enzyme I. The soluble enzyme I was purified by ammonium sulfate fractionation; Bio-Gel P-10 gel filtration; acid precipitation; diethylaminoethyl-Bio-Gel A; and Bio-Gel HTP column chromatography. The enzyme I was shown to be homogeneous by electrophoresis in a pH 8.9 non-sodium dodecyl sulfate gel and by isoelectric focusing. Whereas the protein moved as a single component in both the non-sodium dodecyl sulfate gel and isoelectric focusing, on sodium dodecyl sulfate gels, it moved as three subcomponents. The molecular weights of the three subunits, α, β, and γ, were 44,500, 62,000 and 64,500, respectively. The holoprotein moved as a single component, in the region of 220,000 daltons, in a Bio-Gel A 0.5-agarose column. The molar ratio of subunits was estimated to be 2α:1β:1γ. The elution characteristics on a diethylaminoethyl column at pH 7.4 and 6.8, acid precipitation data, and amino acid composition indicated that the protein is acidic. Isoelectric focusing occurred at pH 4.8. N-terminal amino acids determined by the dansyl chloride method indicated that glycine, alanine, and tyrosine are N-terminal amino acids of the three subunits. Although the protein was stable for at least 14 months at −20°C, it was irreversibly inactivated by the thiol reagent *N*-ethylmaleimide.

The phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) was first reported to occur in mycoplasmas by Van Demark and Plackett (22) and Cirillo and Razin (4). The latter authors reported that the PTS occurred in all of the fermenting species of *Mycoplasma* tested but was absent in the fermenting species of *Acholeplasma*. Subsequent separation and partial characterization of the components of the *M. capricolum* PTS showed that it consists of two general, nonspecific cytoplasmic components, enzyme I and a phosphocarrier protein (HPr), and a sugar-specific, membrane-bound component enzyme II (11).

Enzyme I catalyzes the transfer of the phosphoryl moiety from phosphoenolpyruvate to the phosphocarrier protein HPr as shown:

$$\text{Phosphoenolpyruvate} + \text{HPr} \xrightarrow{Mg^{+2}} \text{P} \sim \text{HPr} + \text{pyruvate}$$

The purification and partial characterization of the *M. capricolum* HPr was reported previously (11). In size (ca. 10,000 daltons) and activity it resembles those of *Escherichia coli* and *Staphylococcus aureus*. In this paper, we report the purification and partial characterization of the *M. capricolum* enzyme I. Although the *M. capricolum* enzyme I shows significant capacity to phosphorylate the *E. coli* HPr (11), it differs from the *E. coli* enzyme in its larger size (220,000 versus 70,000 daltons) and its stability upon purification.

MATERIALS AND METHODS

Organisms and growth conditions. *M. capricolum* Kid was kindly provided by Joseph Tully of the National Institutes of Health, Bethesda, Md. The microorganisms were grown in modified Edwards medium at pH 8.0. The medium contained 0.042 M NaCl, 0.014 M K_2HPO_4, 0.52% (wt/vol) D-glucose, 9.5% (wt/vol) peptone, 0.7% (wt/vol) yeast extract, and 1.3% (wt/vol) heart infusion broth. The medium was supplemented with 20 ml of bovine serum fraction A (Grand Island Biological Co., Grand Island, N.Y.) per liter, 28.0 mg of deoxyribonucleic acid per liter, and 10^6 U of pencillin G (Sigma Chemical Co., St. Louis, Mo.) per liter. The organisms were grown in stationary 2-liter Erlenmeyer flasks containing 500 ml of liquid medium at 37°C. The cells were grown for 24 h. At the end of 16 h, the medium pH was between pH 5.5 and 6.0. At this point, NH_4OH was added asceptically to raise the pH to 7.0. The

cells were harvested when the pH had fallen to 5.5. The culture was rapidly chilled to 0°C, and the cells were harvested by using a Sorvall RC 2-B centrifuge with an HS-4 rotor at 9,000 × *g* for 10 min. Under these growth conditions, the average cell yield ranged from 1.0 to 1.25 g of wet cells per liter of medium. The harvested cells were washed twice with a wash medium containing 0.25 M NaCl and 0.01 M $MgCl_2$. The washed cells were either used directly for fractionation of PTS components or stored at −20°C. All subsequent steps were carried out at 0 to 4°C.

Preparation of crude extracts. Washed cells were suspended in β-buffer [150 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 1 mM ethylenediaminetetraacetic acid, 10 mM β-mercaptoethanol, and 0.1 mM dithiothreitol]. A typical preparation contained 5 mg of wet cells in 20 to 25 ml of β-buffer. Crude enzyme extracts were then prepared from this cell suspension by ultrasonic oscillation and subsequent centrifugation (11).

Separation of membranes and soluble fraction. The crude extracts were recentrifuged at 102,000 × *g* for 90 min at 4°C in a Beckman model L-5-65 ultracentrifuge with a 65 rotor. The supernatant solution was carefully separated and saved for fractionation of enzyme I and HPr. The pelleted membranes were resuspended with a glass homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) and repelleted at 159,000 × *g* for 90 min at 4°C. After this centrifugation, the translucent membrane fraction was suspended in β-buffer and 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 enzyme I. The supernatant fraction obtained by centrifugation at 102,000 × *g* was used for purification of enzyme I; all steps of fractionation were carried out at 0 to 4°C. The steps of purification described below are shown in Table 1, with the indicated specific activity and fold purification achieved at each step.

Step 1: Crude supernatant. The crude supernatant fraction is the soluble material derived from the two high-speed ultracentrifugations described above.

Step 2: Ammonium sulfate precipitation. The crude supernatant fraction from step 1 was adjusted to 100% saturation of $(NH_4)_2SO_4$ by adding crystalline enzyme-grade ammonium sulfate (Schwarz/Mann, Orangeburg, N.Y.) over a 30- to 45-min period with constant stirring in a cold room. The apparent pH of the mixture, measured with a glass electrode, was maintained at 7.4, and the saturated solution was stirred overnight at 4°C. The precipitated proteins were pelleted 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 cm) preequilibrated with β-buffer at a flow rate of about 0.6 ml/min. The column was precalibrated with blue dextran (molecular weight, 2,000,000), cytochrome *c* (molecular weight, 12,400), and *Mycoplasma* HPr (molecular weight, 9,500). The Bio-Gel P-10 gel filtration effectively separated almost all the enzyme I from the HPr. The enzyme I activity was eluted in the void volume, whereas HPr was eluted slightly after cytochrome *c*. The HPr activity eluted from the Bio-Gel P-10 column was totally free from contaminating enzyme I and was the source of HPr (step 3, HPr) in all assays described below.

Step 4: Acid precipitation. The enzyme I fraction from step 3 was adjusted to pH 5.45 by adding ice-cold 0.85 M acetic acid over a 2- to 3-min period with continuous stirring. After 20 min of stirring at 4°C, the precipitate was removed by centrifugation at 30,000 × *g* for 30 min and discarded. Excess 0.85 M acetic acid was added, as described above, until the pH of the enzyme solution dropped to 4.0. The solution was stirred for an additional 30 min at 4°C. The insoluble proteins were pelleted by a brief centrifugation at 30,000 × *g* and suspended in β-buffer.

Step 5: Ammonium sulfate fractionation. The fraction from step 4 was adjusted to 25% saturation by adding crystalline, enzyme-grade ammonium sulfate over a 30-min to 1.0-h period with continuous stirring. During saturation, the apparent pH was maintained at 7.4 by adding ice-cold NH_4OH. After 2 to 3 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 earlier, until 85% saturation was achieved, and the solution was stirred for another 12 h at 4°C. The precipitated proteins were removed by a brief centrifugation at 30,000 × *g*, suspended in 10 to 15 ml of phosphate buffer, and dialyzed against phosphate (diethylaminoethyl [DEAE]-Bio-Gel A buffer, see step 6) for 24 h to remove the ammonium sulfate.

Step 6: First DEAE-Bio-Gel A chromatography (pH 7.4). The dialyzed active fraction (step 5) was applied to a DEAE-Bio-Gel A column (1.5 by 12.5 cm) previously equilibrated with phosphate buffer containing 10 mM potassium phosphate (pH 7.4), 0.1 mM dithiothreitol, and 0.1 mM ethylenediaminetetraacetic acid. After prolonged washing with phosphate buffer, the column was eluted with a linear gradient (0 to 0.3 M) of KCl in potassium phosphate buffer (300 ml total). The enzyme I activity was eluted as a single component at ca. 85 mM KCl, which was coincident with a single protein component (Fig. 1A).

Step 7: Hydroxylapatite column chromatography. The fractions showing enzyme I activity from the DEAE-Bio-Gel A column of step 6 were pooled, dialyzed against potassium phosphate buffer (1 mM potassium phosphate [pH 7.0], 0.1 mM dithiothreitol and 0.1 mM ethylenediaminetetraacetic acid) for 24 h with three changes of buffer, and applied to a hydroxylapatite column (Bio-Gel HTP [1.5 by 10 cm]). The column was preequilibrated with 1.0 mM potassium phosphate buffer (pH 7.0). After prolonged washing, the enzyme I activity was eluted with a 1.0 to 200 mM linear gradient of potassium phosphate buffer (pH 7.0) (300 ml total). The enzyme

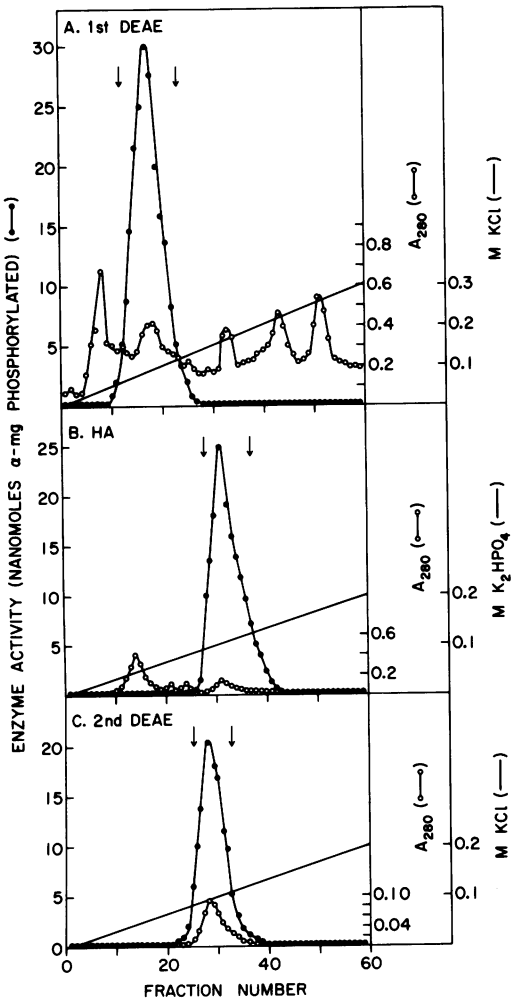


FIG. 1. *Chromatographic purification of enzyme I. In the following chromatographic procedures, the fractions were uniformly of 5.0 ml. (A) First DEAE-Bio-Gel A column (pH 7.4) was eluted with a linear gradient of KCl from 0 to 0.3 M. Only fractions between the arrows were used for hydroxylapatite (Bio-Gel HTP) column. (B) The hydroxylapatite column was eluted with a linear phosphate buffer gradient from 0.001 to 0.2 M phosphate buffer. Only fractions between the arrows were used for second DEAE-Bio-Gel A column. (C) Second DEAE-Bio-Gel A (pH 6.8) column was eluted with a linear KCl gradient from 0 to 0.2 M. The fractions between the arrows were used for further investigation.*

I activity was eluted in a single component at ca. 110 mM potassium phosphate. The enzyme I activity was coincident with a single protein fraction (Fig. 1B).

Step 8: Second DEAE-Bio-Gel A chromatography (pH 6.8). As a final purification step, the active fractions showing enzyme I activity from Bio-Gel HTP column were pooled and dialyzed against a second DEAE-Bio-Gel A column buffer (10 mM potassium phosphate [pH 6.8], 0.1 mM dithiothreitol, and 0.1 mM ethylenediaminetetraacetic acid) for 24 h with three changes of column buffer and applied to a second DEAE-Bio-Gel A column (1.5 by 12.5 cm) preequilibrated with potassium phosphate buffer. After extensive washing with column buffer, the enzyme I activity was eluted with a linear gradient of 0 to 0.2 M KCl in potassium phosphate buffer (pH 6.8) (300 ml total). The enzyme I activity was eluted in a single component at about 95 mM KCl, which was also coincident with a single protein peak (Fig. 1C).

Enzyme assays. The enzyme I activity was measured by an assay system that involves the phosphorylation of α-methyl-D-glucopyranoside (α-MG) in the presence of fixed amounts of HPr and enzyme II. The assay medium is essentially a modification of the Kundig-Roseman medium (13) and the composition is reported elsewhere (11). Briefly, the assay mixture contained 350 to 500 μg of protein of enzyme II and 15 to 25 μg of HPr (step 3). The assay was initiated at 37°C by adding 0.25 μmol of α-[^{14}C]MG (0.5 μCi/μmol) and terminated, usually after 60 min, by heating in boiling water for 4 min. Control incubations contained separate components (i.e., enzyme II or HPr) or lacked enzyme I. The phosphorylated α-MG was separated from the unphosphorylated sugar by passage through a Bio-Rad AG 1-X2 (50- to 100-mesh) anion-exchange resin column (4). The 1 M LiCl eluate, containing phosphorylated α-MG collected directly in a scintillation counting vial, was counted in a scintillation spectrometer (SL-30 Intertechnique, Dover, N.J.) after adding 15.0 ml of scintillation fluid (the composition is reported elsewhere [13]).

The enzyme I and HPr fractions were not significantly contaminated with each other or enzyme II; however, enzyme II preparations (i.e., washed membranes) were constantly contaminated with traces of enzyme I and HPr. Specific activity is expressed as nanomoles of α-MG phosphorylated per milligram of enzyme I protein per minute at 37°C. Since enzyme I activity is coupled to enzyme II activity (via utilization of P ~ HPr as substrate), since these were varied in different experiments, and since different batches of HPr and enzyme II had somewhat different activities, comparisons of specific activity for a given purification regimen of enzyme I required that enzymes from all purification steps (Table 1) be assayed with the same amounts and batch of HPr and enzyme II (11, 21).

Analytical techniques. The protein concentration of soluble enzymes and membrane fractions was determined by the method of Lowry et al. (15), with bovine serum albumin in the appropriate buffer as standard.

Analytical gel filtration was performed on a column (3 by 30 cm) of agarose beads, Bio-Gel A-0.5, equilibrated with 10 mM potassium phosphate buffer (pH 7.4). The sample volumes were 1.0 ml. All protein standards were run individually, and their positions were determined by their absorbances at 280 nm in a Zeiss PMQ II spectrophotometer. Frac-

TABLE 1. *Purification of enzyme I*

Fractionation step	Vol (ml)	Total protein (mg)	Total activity (U)[a]	Relative sp act[b]	Fold purification
1. Crude supernatant	32	500	358	0.71	1
2. Ammonium sulfate: 100% saturation	5.2	495	ND	ND	ND
3. Bio-Gel P-10 gel filtration	32	402	667	1.66	2.3
4. Acid precipitation	10	202	594	2.99	4.0
5. Ammonium sulfate: 25 to 85% saturation	15	148	500	3.36	4.7
6. First DEAE-Bio-Gel A (pH 7.4)	60	21.2	442	20.75	29
7. Hydroxylapatite Bio-Gel HTP	50	1.6	348	216.0	302
8. Second DEAE-Bio-Gel A (pH 6.8)	70	0.52	284	542	761

[a] Nanomoles of α-MG phosphorylated per minute at 37°C; ND, not determined.
[b] Nanomoles of α-MG phosphorylated per milligram of protein per minute at 37°C.

tions eluted from the column (ca. 5.0 ml) were weighed to determine their precise elution volume. The void volume (V_0) and elution volume (V_e) of the column were routinely checked by the elution positions of blue dextran and cytochrome *c*.

Discontinuous gel electrophoresis in non-sodium dodecyl sulfate (SDS) (pH 8.9)-polyacrylamide gels at 7.5% monomer concentration was performed by the method described elsewhere (11). The samples (5 to 10 μg of protein) were subjected to electrophoresis at 4°C with 2.5 mA/gel. The time for the bromophenol blue to move 9.0 cm was ca. 3.75 to 4.0 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).

Electrophoresis in SDS-polyacrylamide gels at 7.5% monomer concentration was performed by the method of Laemmli (14). The samples (2.5 to 5 μg of purified enzyme I protein) were preincubated with a solubilizer containing 2.3% SDS, 15% sucrose, 5% β-mercaptoethanol, and 5 μl of 0.001% bromophenol blue for 5 to 10 min at 100°C. Electrophoresis was carried out at room temperature in a Bio-Rad model 150 gel electrophoresis chamber at 2.5 mA/tube. The running time for the indicator dye to move 9 to 10 cm under these conditions was ca. 4.0 h. The gels were immediately stained for protein with Coomassie brilliant blue (5) and destained in 10% acetic acid.

Isoelectric focusing was performed in polyacrylamide gels by a modification of the method reported by Wrigley (24) and, more recently, by Catterall (3). Gels (5 by 100 mm) were prepared by ammonium persulfate (0.03%, wt/vol)-catalyzed polymerization of a solution containing 7.5% (wt/vol) acrylamide, 0.1% (wt/vol) bisacrylamide, 0.05% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine, and 2.2% (vol/vol) Bio-Lyte (polyaminopolysulfonic acid) 3-10. Samples (150 μl) were made in 10% (vol/vol) sucrose and 0.1% (vol/vol) Bio-Lyte 3-10. After the application of the samples, the gel tubes were filled with cathode buffer (100 mM NaOH) and immersed in anode buffer (10 mM phosphoric acid) in a Bio-Rad model 150 gel electrophoresis chamber with a water jacket through which cold water was circulated during the experiment. The protein was focused at room temperature. An electric field of 50 V was applied initially for 30 min, the electric field was later increased to 150 V, and the protein was focused for 5.0 h, after which the gels were immediately fixed by immersion in ice-cold 10% (wt/vol) trichloroacetic acid. The gels were dialyzed against 10% trichloroacetic acid for 24 h to remove carrier Bio-Lytes and stained with Coomassie brilliant blue by the method of Fairbanks et al. (5).

Amino acid composition. Amino acid analyses were carried out with an automatic amino acid analyzer (JEOL-JLC-6AH). For this purpose, 25 to 50 μg of purified enzyme I was initially lyophilized. To estimate the amide content, the following treatment was performed: a 0.5-ml portion of 0.2 N borate buffer (pH 9.0) was added to the lyophilized enzyme I, boiled for 10 min, and dried in a desiccator over concentrated H_2SO_4 as the desiccant (10). Hydrolysis of enzyme I was then carried out in a sealed, evacuated tube in 0.4 ml of constantly boiling HCl (Pierce Chemical Co., Rockford, Ill.) for 24 h at 110°C. A performic acid oxidation was carried out to determine cysteine content by the method of Hirs (9). Tryptophan content was determined by the method of Goodwin and Morton (6).

N-terminal determination. The N-terminal residues of enzyme I were determined by the 5-dimethylaminonaphthalene-1-sulfonyl (DNS)-chloride procedure (7). For this purpose, 5 nmol of enzyme I (molecular weight, 220,000) was lyophilized. The lyophilized protein was dissolved in 10 μl of 0.2 M $NaHCO_3$ and relyophilized. This step was repeated. The thrice-lyophilized protein was dissolved in 10 μl of deionized water to which 10 μl of DNS-Cl (2.5 mg/ml in acetone) was added. The tube containing protein in the DNS-Cl solution was sealed with Para-

film and incubated for 1.0 h at 37°C. After dansylation, the protein was lyophilized, and 50 μl of 6.0 N HCl was added, and the mixture was sealed and incubated for 18 h at 105°C. The hydrolyzed, dansylated protein was lyophilized, and 10 μl of 50% (vol/vol) pyridine was added to the tube; 0.1-μl proteins were spotted out on polyamide thin-layer sheets (15 by 15 cm; Cheng Chin Trading Co. Ltd., Taiwan). A 1.5-μl sample was spotted per polyamide sheet. Thin-layer chromatography was run for 50 min in solvent 1 (1.5% [vol/vol] formic acid), dried, run for 1.0 h in solvent 2 (benzene–acetic acid, 9:1, vol/vol), dried, and run for 1.0 h in solvent 3 (ethyl acetate–methanol–acetic acid; 20:1:1, vol/vol/vol). The chromatography was performed in an Eastman chromagram-developing apparatus 6071 (Eastman Chemical Products, Inc., Kingsport, Tenn.). Appropriate markers (i.e., DNS-Gly, DNS-Ala, DNS-Leu, DNS-Ileu, DNS-Val, DNS-Thr, DNS-Ser, DNS-Trp, DNS-Glu, DNS-Asp, DNS-Pro, DNS-Arg, DNS-Lys, DNS-Phe, *O*-DNS-Tyr, and bis-DNS-Tys) were run identically in polyamide layers. The position of the DNS derivatives was visualized with an ultraviolet lamp.

Source of materials. Phosphoenolpyruvate-tricyclohexamine salt, dithiothreitol, DNS-Cl, and dansylated amino acid markers were purchased from Sigma, and bovine serum fraction A (IX) was purchased from the Grand Island Biological Co., Grand Island, N.Y. Anion-exchange resin AG 1-X2 (50 to 100 mesh), Bio-Gel P-10 polyacrylamide beads, Bio-Gel A-0.5 agarose beads, anion exchanger DEAE-Bio-Gel A, hydroxylapatite Bio-Gel HTP, materials for polyacrylamide gel electrophoresis, isoelectric focusing in polyacrylamide gels, and Bio-Lyte carrier ampholyte (pH 3 to 10) were purchased from Bio-Rad Laboratories, Richmond, Calif. α-[*U*-^{14}C]MG was purchased from Amersham/Searle, England. All other chemicals were obtained from available commercial sources and were of analytical or reagent grade.

RESULTS

Purification. A scheme basically similar to the one used for the purification of the phospho-carrier protein of *Mycoplasma* (11) was utilized to purify enzyme I. The steps used were ammonium sulfate fractionation, acid precipitation, gel filtration, adsorption, and anionic-exchanger column chromatography. The results of the successive purification steps described above are shown in Table 1 and Fig. 1. The eight-step purification produced a 761-fold-purified preparation with a specific activity of 542 nmol of α-MG phosphorylated per min per mg of protein. The final product eluted from the second DEAE-Bio-Gel A column was shown to be homogeneous by both polyacrylamide gel electrophoresis and isoelectric focusing in polyacrylamide gels (Figs. 2B and 3).

Stability, absorption spectrum, and molar extinction coefficient. The protein was found

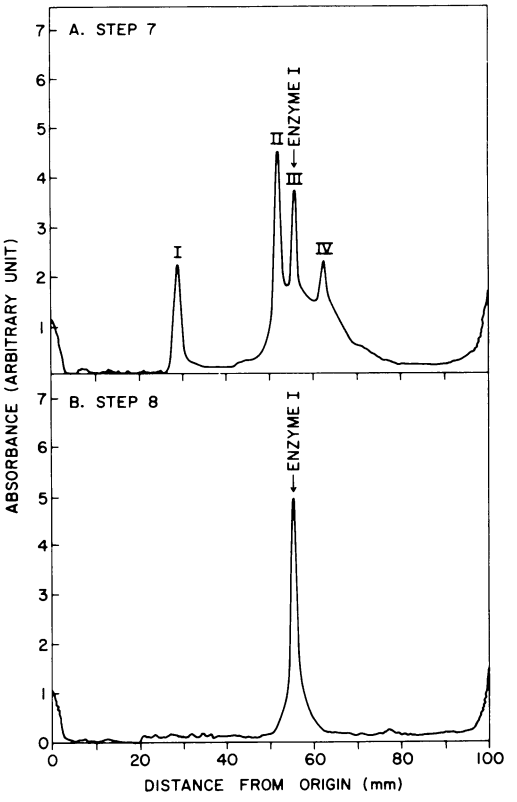


FIG. 2. *Densitometer tracing of stained pH 8.9 non-SDS gels. (A) Level of purification, step 7. (B) Level of purification, step 8.*

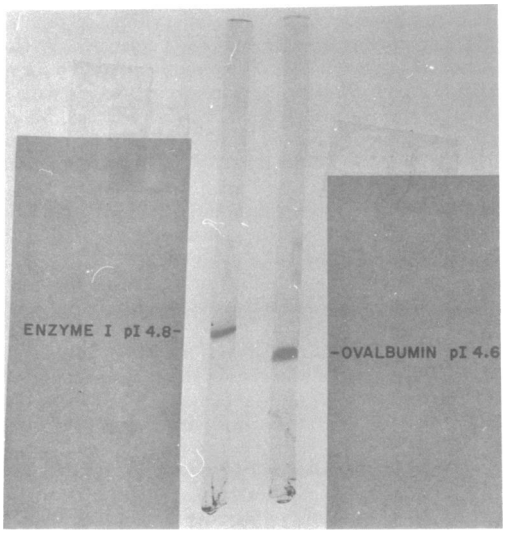


FIG. 3. *Stained purified enzyme I and ovalbumin focused at their isoelectric points in polyacrylamide gels.*

to be remarkably stable. The purified protein (step 7) was found to be stable for at least 14 months when stored at −20°C in phosphate buffer (pH 7.0) at a protein concentration of 0.1 mg/ml. The absorption spectrum of *Mycoplasma* enzyme I is typical of proteins. The estimated absorbance at 280 nm of the enzyme at a concentration of 1.0 mg/ml (1.0-cm light path) was 1.05, and the 280/260 absorbance ratio was 1.4. Based on a molecular weight of 220,000 (see below), the $\epsilon_M \times 10^{-4}$ at 280 nm is 23.1.

Homogeneity. The purified enzyme moved as a single component during electrophoresis in polyacrylamide gels at a 7.5% monomeric concentration, (pH 8.9, Fig. 2B), and the spacer gel was clear. A comparison of the protein profile during purification steps 7 and 8 showed that, although, in step 7, enzyme I was contaminated with three extraneous bands, the second DEAE column (step 8) successfully separated enzyme I from the rest of the contaminating proteins (Fig. 2A, B). The active fractions from the second DEAE column were individually tested for the presence of enzyme I by gel electrophoresis at pH 8.9; all the fractions (27 through 34) showed one band corresponding to enzyme I. However, the trailing part of the enzyme I in the second DEAE column (beyond 35) showed a minor contaminating protein (data not presented). We analyzed individual fractions by gel electrophoresis to determine which fractions to include for further characterization.

As an additional test for homogeneity, the purified protein was subjected to polyacrylamide gel isoelectric focusing by using carrier ampholyte Bio-Lyte (pH 3 to 10). The protein migrated as a single, sharp band (Fig. 3).

Molecular weight. The molecular weight of enzyme I was estimated by the technique of analytical gel filtration in Bio-Gel A-0.5 agarose beads. The relative elution position of enzyme I, along with other proteins of known molecular weight (i.e., apoferritin, aldolase, ovalbumin, and others), is shown in Fig. 4. Enzyme I was eluted between apoferritin and aldolase, and the apparent molecular weight was estimated to be ca. 220,000.

Quaternary structure. Enzyme I from *Mycoplasma* could be dissociated with SDS in the presence of dithiothreitol and β-mercaptoethanol into three subunits as revealed by electrophoresis in polyacrylamide gels (7.5% monomeric concentration) in the presence of 0.1% SDS (Figs. 5, 6). The relative mobility of the subunit species in relation to the migration of protein standards of known molecular weight indicated the molecular weights of the subunits, α, β, and γ, to be 44,500, 62,000, and 64,500,

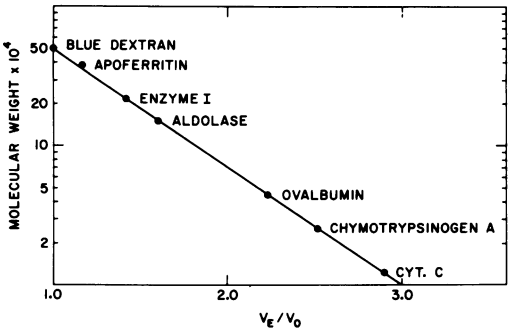


FIG. 4. *Analytical gel filtration of enzyme I and protein standards in Bio-Gel A-0.5 agarose beads.*

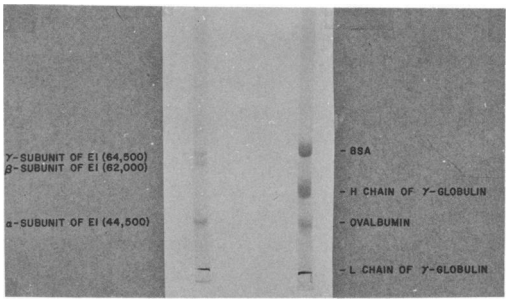


FIG. 5. *SDS-polyacrylamide gel electrophoresis of enzyme I. Purified enzyme I was subjected to discontinuous gel (7.5%) electrophoresis in 0.1% SDS.*

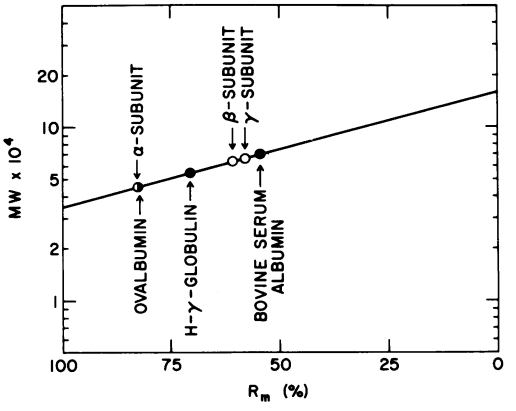


FIG. 6. *Relative mobility of enzyme I subunits in SDS gel along with protein standards.*

respectively, with an experimental error as high as ±10% (23). The densitometric tracing of stained SDS gels indicate a α/β/γ molar ratio of 2:1:1 (data not shown). The estimated molecular weight of the holoprotein on the basis of this subunit ratio is ca. 215,500 ± 21,500. The calculated molecular weight of enzyme I agrees with

the molecular weight estimated by analytical gel filtration in the Bio-Gel A-0.5 column (Fig. 4).

N-terminal determination. The N-terminal amino acids of enzyme I were determined by polyamide thin-layer chromatography of the hydrolyzed, dansylated protein (7).

Fig. 7 depicts the separated, dansylated N-terminal amino acids of enzyme I. Six strong fluorescent spots were detected on the polyamide sheets: DNS-OH, DNS-NH_2, *O*-DNS-Tyr, DNS-Ala, DNS-Gly, and bis-DNS-Tyr. Among these, the last three (i.e., DNS-Ala, DNS-Gly, and bis-DNS-Tyr) represent the N-terminal amino acid residues of the three subunits (Fig. 7).

Amino acid composition. The results of the amino acid analyses of enzyme I are summarized in Table 2. The protein contained a high amount of aspartic and glutamic acids (528 residues) as compared to 244 basic amino acid residues. The protein contained 26 cysteine residues and was found to be low in tryptophan (12 residues). The molar ratio of tyrosine to tryptophan as determined by the spectrophotometric method of Goodwin and Morton (6) was 4.04.

Isoelectric pH determination. Isoelectric pH of the purified enzyme I holoprotein was determined by isoelectric focusing in polyacrylamide gels. The protein focused at ca. pH 4.8 and, under identical conditions, ran close to ovalbumin, pI 4.6 (Fig. 3).

Sensitivity toward thiol reagent. The sensitivity of enzyme I, HPr, and enzyme II to the sulfhydryl reagent *N*-ethylmaleimide (NEM) is shown in Table 3. Essentially, 100% of the phosphotransferase activity was inhibited when NEM was added to either enzyme I or HPr. The addition of β-mercaptoethanol 20 min after the addition of NEM to HPr, enzyme I, and enzyme II reduced the inhibition to 0, 91.5, and 66%, respectively. Thus, the NEM inhibition of en-

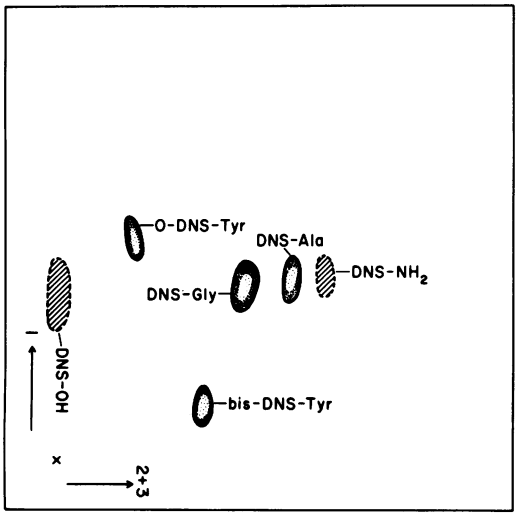


FIG. 7. *Two-dimensional, thin-layer chromatogram (polyamide layer) of DNS-amino acids derived from dansylation of purified enzyme I. Number and arrow indicate the solvent and direction of run.*

TABLE 2. *Amino acid analysis of Mycoplasma enzyme I*

Amino acid residue	Residue[a]/27.5 μg of protein	Nearest integral no. of residues per molecule of protein of mol wt 215,500
Lysine	5.46	150
Histidine	1.27	35
Arginine	2.15	59
Aspartic acid	8.82	242
Threonine	3.92	108
Serine	5.27	145
Glutamic acid	10.4	286
Proline	3.12	86
Glycine	6.3	173
Alanine	4.28	118
Valine	3.88	107
Methionine	1.65	45
Isoleucine	4.63	127
Leucine	6.38	175
Tyrosine	1.78	49
Phenylalanine	3.01	83
Cysteine[b]	0.94	26
Tryptophan[c]	0.44	12
Ammonia	3.01	83

[a] Experimental procedure described in the text.
[b] Determined as cysteic acid (9).
[c] Determined spectrophotometrically (6).

TABLE 3. *Sensitivity of Mycoplasma HPr, enzyme I, and enzyme II to NEM*[a]

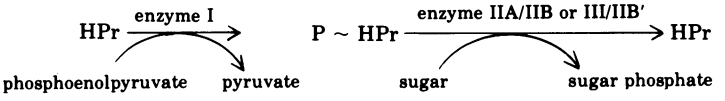
Fraction treated with NEM	β-Mercaptoethanol addition	α-MG phosphorylated (nmol)	PTS activity (% activity of control)
HPr	20 min after NEM	130.16	107
Enzyme I	20 min after NEM	7.67	8.2
Enzyme II	20 min after NEM	59.0	33.9
HPr	Not added	0	0
Enzyme I	Not added	0	0

[a] In this experiment 25 μg of HPr (step 3) in 50 μl of 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid and 0.1 mM dithiothreitol was treated with 20 μl of 0.1 M sulfhydryl reagent. After 20 min at room temperature, 20 μl of 0.2 M β-mercaptoethanol was added. A 75-μg portion of enzyme I in 50 μl of potassium phosphate buffer and 112 μg of crude enzyme II in 50 μl of β-buffer were similarly treated with NEM and β-mercaptoethanol. In control experiments, 20 μl of sulfhydryl reagent was first mixed with 20 μl of β-mercaptoethanol and then added to HPr, enzyme I, and enzyme II fractions. PTS assays were carried out by the procedure outlined in the text.

zyme I was irreversible, whereas the inhibition of HPr and enzyme II was completely or partially reversed by β-mercaptoethanol.

DISCUSSION

The phosphoenolpyruvate-dependent sugar phosphotransferase systems studied consist of four types of proteins: two general, nonspecific proteins that are always cytoplasmic (enzyme I and HPr) and two sugar-specific proteins that may be membrane bound (IIA/IIB) or may consist of one soluble (III) and one membrane-bound component (IIB') (1, 12, 17, 18, 20). The system carries out the following reaction:



The sugar phosphorylation mediated by the membrane-bound components (IIB or IIB') is linked to sugar transport across the cell membrane (18).

In mycoplasmas, enzyme I and HPr are the only soluble components (11). The membrane-bound sugar-specific enzyme II activities for glucose, fructose, and mannose are, therefore, presumed to be of the IIA/IIB type. In *M. capri*, the enzymes II for the three sugars are constitutive (4); in *M. capricolum*, the enzyme II for fructose is inducible (unpublished data). The purification and partial characterization of the *M. capricolum* HPr were previously described (11). It is similar to the *E. coli* and *S. aureus* phosphocarrier proteins in size, and its phosphorylated form, P ~ HPr, is equally as active as a phosphoryl donor with *E. coli* enzyme IIA/IIB complex as the *E. coli* P ~ HPr. However, it is different from the *E. coli* HPr in amino acid composition, electrophoretic characteristics, immunological reactivity, and as a substrate for the *E. coli* enzyme I (11).

In the present paper, we report the purification and partial characterization of *M. capricolum* enzyme I. The physical characteristics of the *Mycoplasma* enzyme are quite distinct from those of *E. coli*, *Salmonella typhimurium*, and *S. aureus*, the only other enzymes that have been purified and characterized (enzyme I of *E. coli* and *S. typhimurium* are identical [17]). The *E. coli*/*S. typhimurium* enzyme has a molecular weight of between 70,000 and 90,000, with an unknown number of subunits (21); the *S. aureus* enzyme is a single polypeptide chain with a molecular weight of 80,000 (8). The *Mycoplasma* holoenzyme has a molecular weight of ca. 220,000 and is composed of four subunits: two α of 44,500 daltons, one β of 62,000 daltons, one γ of 64,500 daltons. The suggestion that there are two α subunits is based on the following data: (i) the molecular weight estimate of the holoenzyme by analytical gel filtration required two α subunits and (ii) the intensity of the α band in Coomassie brilliant blue-stained SDS gels was roughly twice that of β and γ bands. The identifications of only three N-terminal amino acids by the dansyl procedure requires, therefore, that the two α subunits be identical, but this is only speculation, with the limited data at hand.

The *Mycoplasma* enzyme I is more stable than the *E. coli* and *S. aureus* enzymes I. The stability of the purified *Mycoplasma* enzyme may be due to its complex structure; the presence of the additional subunits may protect the enzyme from inactivation during purification. However, the *Mycoplasma* enzyme is as sensitive to NEM as the *E. coli* and *S. aureus* enzymes (17). This sensitivity is understandable, considering the presence of 25 to 26 cysteines in the molecule; however, at this point, we do not know how they are distributed among the subunits.

Complementation studies between *Mycoplasma* and *E. coli* enzymes I and HPr's show that there is considerable similarity between the two systems. Thus, the *Mycoplasma* enzyme I phosphorylates the *E. coli* HPr, and the *E. coli* enzyme I phosphorylates the *Mycoplasma* HPr, although the rate in each case is reduced to ca. 20% of the rate obtained with homologous components (11). This represents a significant overlap of activity in spite of the large size difference between the *Mycoplasma* and *E. coli* enzymes. This may suggest that the four subunits of the *Mycoplasma* holoenzyme represent a complex in which only one of the subunits bears the enzyme I activity and the other subunits serve regulatory or other functions. Support for this proposal will be sought in future studies of the nature and distribution of phosphoryl groups among the enzyme I subunits. Evidence that enzyme I undergoes a phosphorylation-dephosphorylation cycle in the phosphoryl transfer from phosphoenolpyruvate to HPr has been presented for some of the enzymes studied (17, 20). One function of enzyme I that might be served by its noncatalytic subunits is interaction with adenylate cyclase, as proposed by Peterkofsky and Gazdar (16) to explain the effect of PTS sugar substrates on

adenylate cyclase activity in *E. coli*. It is known that, in mycoplasmas, the intracellular concentration of cyclic adenosine 3'5'-monophosphate is subject to regulation by the presence of glucose in the medium in a manner similar to that in *E. coli* (unpublished data). Peterkofsky and Gazdar proposed that the level of cellular cyclic adenosine 3'5'-monophosphate in *E. coli* is regulated by the level of phosphorylated enzyme I that is required for adenylate cyclase activity. If such a mechanism were involved in mycoplasmas, noncatalytic subunits would be required for enzyme I-adenylate cyclase interaction. However, a direct involvement of enzyme I is not favored by some workers who propose that the interacting species between the PTS and adenylate cyclase is the glucose-specific factor III^{glc} rather than enzyme I (2, 19). Which, if either, of these proposals applies to *Mycoplasma* is now under investigation.

ACKNOWLEDGMENTS

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

We happily acknowledge the technical assistance of Stuart Kozinn and Stuart Weisman.

LITERATURE CITED

1. **Anderson, B., N. Weigel, W. Kundig, and S. Roseman.** 1971. Sugar transport. III. Purification and properties of a phosphocarrier protein (HPr) of the phosphoenopyruvate-dependent phosphotransferase system of *E. coli*. J. Biol. Chem. 246:7023-7033.
2. **Castro, L., B. U. Feucht, M. L. Morse, and M. H. Saier, Jr.** 1976. Regulation of carbohydrate permeases and adenylate cyclase in *E. coli*. J. Biol. Chem. 251:5522-5527.
3. **Catterall, W. A.** 1976. Purification of a toxic protein from scorpion venom which activates the action potential Na^+ ionophore. J. Biol. Chem. 251:5528-5536.
4. **Cirillo, V. P., and S. Razin.** 1973. Distribution of a phosphoenolpyruvate-dependent sugar phosphotransferase system in mycoplasmas. J. Bacteriol. 113:212-217.
5. **Fairbanks, G., T. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606-2617.
6. **Goodwin, T. W., and R. A. Morton.** 1946. The spectrophotometric determination of tyrosine and tryptophan in protein chemistry. Biochem. J. 40:628-632.
7. **Hartley, B. S.** 1970. Strategy and tactics in protein chemistry. Biochem. J. 119:805-822.
8. **Hengstenberg, W.** 1976. *In* Proceedings of the 3rd International Symposium on Staphylococci and Staphylococcal Infections. Gustav Fischer Verlag, Stuttgart.
9. **Hirs, C. H. W.** 1956. The oxidation of ribonuclease with performic acid. J. Biol. Chem. 219:611-621.
10. **Inouye, M., Y. Okada, and A. Tsugita.** 1970. The amino acid sequence of T4 phage lysozyme. J. Biol. Chem. 245:3439-3454.
11. **Jaffor Ullah, A. H., and V. P. Cirillo.** 1976. *Mycoplasma* phosphoenolpyruvate-dependent sugar phosphotransferase system: purification and characterization of the phosphocarrier protein. J. Bacteriol. 127:1298-1306.
12. **Kundig, W., S. Ghosh, and S. Roseman.** 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. Proc. Natl. Acad. Sci. U.S.A. 52:1067-1074.
13. **Kundig, W., and S. Roseman.** 1971. Sugar transport II. Characterization of constitutive membrane bound Enzyme II of the *E. coli* phosphotransferase system. J. Biol. Chem. 246:1407-1418.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
16. **Peterkofsky, A., and C. Gazdar.** 1975. Interaction of enzyme of the phosphoenol pyruvate: sugar phosphotransferase system with adenyl cyclase of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. 72:2920-2924.
17. **Postma, P. W., and S. Roseman.** 1976. The bacterial phosphoenolpyruvate: sugar phosphotransferase system. Biochim. Biophys. Acta 457:213-257.
18. **Roseman. S.** 1969. The transport of carbohydrates by a bacterial phosphotransferase system. J. Gen. Physiol. 54:138s-184s.
19. **Saier, M. H., Jr., B. U. Feucht, and L. J. Hofstadter.** 1976. Regulation of carbohydrate uptake and adenylate cyclase activity mediated by the enzymes II of the phosphoenolpyruvate: sugar phosphotransferase system in *E. coli*. J. Biol Chem. 251:883-892.
20. **Simoni, R. D.** 1972. Macromolecular characterization of bacterial transport system, p. 289-322. *In* C. F. Fox and A. D. Keith (ed.), Membrane molecular biology. Sinauer Associates, Inc., Stamford, Conn.
21. **Simoni, R. D., T. Nakazawa, J. B. Hays, and S. Roseman.** 1973. Sugar transport IV. Isolation and characterization of the lactose phosphotransferase system in *Staphylococcus aureus*. J. Biol. Chem. 248:932-940.
22. **Van Demark, P. J., and P. Plackett.** 1972. Evidence for a phosphoenolpyruvate-dependent sugar phosphotransferase in *Mycoplasma* strain Y. J. Bacteriol. 111:454-458.
23. **Weber, K., and M. Osborn.** 1969. Reliability of molecular weight determinations by dodecylsulfate polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.
24. **Wrigley, C. W.** 1968. Analytical fractionation of plant and animal proteins by gel electrofocusing. J. Chromatogr. 36:362-365.