Expression, Purification, and Characterization of Enzyme IIA^{glc} of the Phosphoenolpyruvate:Sugar Phosphotransferase System of *Mycoplasma capricolum*

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ABSTRACT: The gene encoding enzyme IIA^{glc} (EIIA) of the phosphoenolpyruvate:sugar phosphotransferase system of *Mycoplasma capricolum* was cloned into a regulated expression vector. The purified protein product of the overexpressed gene was characterized as an active phosphoacceptor from HPr with a higher p*I* than previously described EIIAs. *M. capricolum* EIIA was unreactive with antibodies directed against the corresponding proteins from either Gram-positive or Gram-negative bacteria. Enzyme IIA^{glc} behaved as a homogeneous, monomeric species of 16 700 M_r in analytical ultracentrifugation. The circular dichroism far-UV spectrum of EIIA reflects a low α-helical content and predominantly β-sheet structural content: temperature-induced changes in ellipticity at 205 nm showed that the protein undergoes reversible, two-state thermal unfolding with $T_m = 70.0 \pm 0.3$ °C and a van't Hoff ΔH of 90 kcal/mol. Enzyme I (64 600 M_r) from *M. capricolum* exhibited a monomer−dimer−tetramer association at 4 and 20 °C with dimerization constants of log $K_A = 5.6$ and 5.1 [M^{-1}], respectively, in sedimentation equilibrium experiments. A new vector, capable of introducing an N-terminal His tag on a protein, was developed in order to generate highly purified heat-stable protein (HPr). No significant interaction of EIIA with HPr was detected by gel-filtration chromatography, intrinsic tryptophanyl residue fluorescence changes, titration calorimetry, biomolecular interaction, or sedimentation equilibrium studies. While *Escherichia coli* EIIA inhibits Gram-negative glycerol kinase activity, the *M. capricolum* EIIA has no effect on the homologous glycerol kinase. The probable regulator of sugar transport systems, HPr(Ser) kinase, was demonstrated in extracts of *M. capricolum* and *Mycoplasma genitalium*. Gene mapping studies demonstrated that, in contrast to the clustered arrangement of genes encoding HPr and enzyme I in *E. coli*, these genes are located diametrically opposite in the *M. capricolum* chromosome.

Mycoplasmas are organisms of fundamental interest since they constitute the class of the smallest free-living organisms with the least complex genomes (*1*). The entire sequence of the genome of *Mycoplasma genitalium* has recently been reported (*2*). This laboratory has been analyzing the genome of *Mycoplasma capricolum*, with an emphasis on the genes encoding proteins involved with sugar transport and regulation (*3*, *4*). The phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] promotes phosphotransfer from phosphoenolpyruvate to the heat-stable protein, HPr, in a reaction catalyzed by enzyme I (*5*). The phosphorylated form of HPr is capable of participating in phosphotransfer reactions to a variety of sugar-specific acceptor proteins, termed enzymes II. The enzymes II ultimately participate in the coordinated transport and phosphorylation of specific sugar substrates of the PTS.

In order to characterize enzyme IIA^{glc}, the structural gene has been cloned into a hyperexpression vector and the overproduced protein has been purified and characterized. An important finding is that, in contrast to the homologous protein from *Escherichia coli*, which regulates glycerol kinase, the *Mycoplasma* EIIA does not show such regulation. The demonstration of the presence of HPr(Ser) kinase (*6*) in *Mycoplasma* extracts supports a model that sugar transport regulation in mycoplasmas follows the same pattern as in Gram-positive bacteria (*7*).

MATERIALS AND METHODS

Microbiological Methods

M. capricolum (kid strain) was grown in modified Edwards medium at pH 8 as described previously (*4*). *M. genitalium* was grown as previously described (*2*). Plasmids were introduced into *E. coli* GI698 by electroporation as previously described (*8*).

DNA Methods

Construction of Vector pMC-IIA. The DNA sequence from base 4317 to 5402 of the published sequence (*4*) encoding *M. capricolum* EIIA was amplified using pPZ6 as a template. The forward PCR primer (5′-AGGTGCCATATGTGGTTTTTTAATAAG-3′) contained an engineered *Nde*I site (underlined). Since the second codon of the *M. capricolum* EIIA corresponds to Trp in mycoplasma but to

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[1] Abbreviations: DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; PTS, phosphoenolpyruvate:sugar phosphotransferase system; *crr*, the gene encoding enzyme IIA^{glc} of the PTS; EIIA, enzyme IIA^{glc}; CD, circular dichroism; FIGE, field inversion gel electrophoresis; ENase(s), restriction endonuclease(s); PolIK, the Klenow fragment of DNA polymerase I.

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termination in *E. coli*, that codon was mutated to an *E. coli* Trp codon for efficient expression in *E. coli*. The reverse PCR primer (5′-CTTCTTTAACATTATCTAGATTTAA-ACTCA-3′) contained an engineered *Xba*I site (underlined). The PCR product was cloned into the *Nde*I and *Xba*I cloning sites of the vector pREI (*9*) and the recombinant plasmid was transformed into strain GI698 (*8*). DNA sequence analysis by the dideoxy method of Sanger et al. (*10*) was used to verify the constructs.

Construction of Vector pRE-His-Tag. Plasmid pREI (*9*) was digested with *Mun*I and *Nde*I. A pair of complementary primers (5′-AATTGTTATCTAAGGAAATACTTACAA**AT-GCACCATCACCATCACCA**-3′ and 5′-T**ATGGTGATG-GTGATGGTGCAT**TTGTAAGTATTTCCTTAGATAAC-3′) (location of the restriction sites underlined) were designed to place a methionine followed by a cluster of six histidine residues (shown in boldface type) before the normally occurring start codon. The annealed oligonucleotides were ligated to the digested pREI and transformed into strain DC646 (*9*); transformants were screened by PCR and confirmed by DNA sequencing.

Southern Blotting. *M. capricolum* chromosomal DNA, prepared by the agarose bead method (*11*−*13*), was digested with ENases and separated by FIGE run in 1% agarose, at 6.2 V/cm, for 18 h with pulse time ramping from 3 to 20 s at ratio 3 (*14*). The separated DNA fragments were probed with ^{32}P-labeled probes for *ptsH* and *ptsI*−*crr* as described previously (*3*, *4*). The *ptsH* probe was the 695 bp *Hin*dIII fragment excised from plasmid pPZ1 (*4*). The *ptsI*−*crr* probe was made by PCR amplification of a 256 bp region containing sequences within the *kdtB* and *ptsI* genes (*4*), which were modified to contain *Spe*I sites. Prehybridization was performed in 6× SSPE, 1% SDS, and 10× Denhardt's solution containing 10 μg/mL tRNA and 100 μg/mL denatured heterologous DNA at 65 °C for 2.5 h. Hybridization was performed in prehybridization solution containing (0.8−1.4) × 10^6 cpm of ^{32}P-labeled DNA probe/mL at 65 °C for 16 h. The blots were finally washed in 0.5× SSPE and 1% SDS solution at 37 °C for 0.5 h.

Protein Methods

Expression and Purification of His-Tagged HPr. Strain GI698 transformed with pMC-His-Tag-HPr was grown in synthetic medium and processed through the stage of the 100000*g* supernatant as previously described (*8*). The supernatant fraction was purified on Ni-NTA-agarose (Qiagen). The fraction from Ni-NTA-agarose was chromatographed on a MonoQ HR 10/10 (FPLC, Pharmacia) column. The His-tagged HPr did not bind to the column and was collected in the wash (25 mM Tris·HCl, pH 7.5). Protein concentrations were determined by the method of Waddell (*15*) or in the case of EIIA by UV absorbance measurements at 280 nm ($A^{0.1\%}_{280nm,1cm}$ = 0.348). SDS−PAGE was carried out according to Laemmli (*16*). Densities of proteins in stained gels and autoradiograms were quantitated by using the NIH IMAGE program (version 1.55) develped at the National Institutes of Health by Wayne Rasband.

Circular Dichroism. CD spectra were measured with a Jasco J-710 spectropolarimeter using a water-jacketed cylindrical cell with 0.05 cm optical path length and an external programmable water bath. Spectra were corrected for the solvent CD signal. For determining secondary structure, EIIA spectra were the average of 4 accumulations taken at 10 nm/min and have been smoothed using a fast Fourier transform algorithm. Data are reported as mean residue ellipticities (calculated mean residue mol wt = 108.46). Secondary structural components were calculated (*17*) from far-UV CD spectra. For thermal denaturation studies, temperature was increased at the rate of 30, 60, and 90 °C/h. Data were analyzed by a two-state thermodynamic analysis program (*18*).

Analytical Ultracentrifugation. A Beckman Optima Model XL-A ultracentrifuge (Beckman Instruments, Inc.) equipped with a four-place titanium rotor was used at 4 and 20 °C. Buffer densities at 20 ± 0.01 °C (ρ_{20}) were determined with an Anton Paar Model DMA-58 densitometer. Sedimentation equilibrium experiments were conducted using a carbon-filled, 6-channel centerpiece in a 12-mm cell. Increasing concentrations of each protein were loaded (0.120 mL/channel) with 0.130 mL of reference buffer/channel: 0.14, 0.28, and 0.50 mg/mL EIIA, 0.06, 0.12, and 0.24 mg/mL HPr, and 0.28, 0.49, and 0.90 mg/mL enzyme I. Dialysis buffers were 100 mM K·PO_4, 1 mM EDTA, and 2 mM 2-ME, pH 7.5 (ρ_{20} = 1.0122) for EIIA and HPr and 10 mM K·PO_4, 100 mM KCl, 1 mM EDTA, and 2 mM 2-ME, pH 7.5 (ρ_{20} = 1.0046), for enzyme I. Partial specific volumes of 0.728, 0.727, and 0.728 mL/g for EIIA, HPr, and enzyme I, respectively, were calculated from amino acid compositions derived from DNA sequences (*3*, *4*) and the values of Zamyannin (*19*). Rotor speeds of 19 000 and 22 000 rpm for EIIA, 29 000 rpm for HPr, and 11 000 rpm for enzyme I were used. Radial scans (0.001 cm step mode) were conducted at 280 nm for EIIA (± HPr), 237 nm for HPr (which contains no Trp, Tyr, or Cys) and 282 nm for enzyme I. Equilibrium was reached within 24−48 h at each speed and temperature, as confirmed when radial scans made at 4 h intervals could be superimposed. Analysis of sedimentation equilibrium data included buffer baseline corrections (<0.03) and weighted fits for the instrument noise. These analyses as well as global fitting to various models for data obtained at the three concentrations in each case were performed with the software provided by Allen P. Minton (NIDDK, NIH) which can be downloaded from the following World Wide Web site: http://bbri-www.eri.harvard.edu/RASMB/rasmb.html.

HPr−*EIIA Interaction Measurements*. (i) Fluorescence quenching: A SLM-Aminco-Bowman Series 2 fluorometer (Spectronic Instruments, Inc.) was used for titration of EIIA with HPr with excitation and emission at 295 and 349 nm, respectively. (ii) Titration calorimetry: HPr was added to EIIA in a titration calorimeter (MCS-ITC) (MicroCal,Inc.) as previously described (*20*). (iii) Biomolecular interaction analysis: Tests for interaction of HPr and EIIA used surface plasmon resonance on a BIAcore instrument (Pharmacia Biosensor). Purified HPr coupled to a CM-5 sensor chip was tested for interaction with concentrations of EIIA ranging from 5 to 10 mg/mL. (iv) Sedimentation equilibrium: Mixtures of EIIA with HPr were run as above for EIIA alone. (v) Gel-filtration chromatography: Equimolar mixtures of EIIA and His-tagged HPr (150 μM) and BSA (25 μM) were chromatographed on a Sephadex G-75 superfine (0.9 × 52 cm) column which was equilibrated and eluted with His-tagged HPr (50 μM) in 25 mM Tris·HCl, pH 8.0. The column was run at 4 °C and fractions of 250 μL were collected. Fluorescence in the fractions was detected at an excitation wavelength of 295 nm and an emission wavelength of 360 nm.

Phosphoacceptor Activity of Enzyme IIA^{glc}. Phosphotransfer from phosphoenolpyruvate to EIIA was carried out in a reaction mixture (10 μL) containing 100 mM K·PO_4, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.078 unit of pyruvate kinase, 1 mM pyruvate, 0.2 mM ATP ([γ-^{32}P]ATP, 50 000 cpm/nmol), 0.15 μg of *E. coli* HPr, 0.15 μg of *E. coli* enzyme I, and 2 μg of *M. capricolum* enzyme IIA^{glc}; incubation was at room temperature for 10 min. The mixture was then electrophoresed on an SDS−polyacrylamide gel (4−20% Tris·glycine gradient Novex mini-gel). The gel was then stained with Coomassie blue and dried. The dried gel was exposed to Kodak XAR film.

Glycerol Kinase Assay. Frozen cells of *M. capricolum* were mixed with 20 mM Tris·HCl (20% w/v) and sonicated to break the cells. The sonicate was centrifuged for 30 min at 14000*g* and the supernatant solution was used as the crude extract for glycerol kinase assay. The assay was carried out as described previously (*21*) using 50 mM HEPES buffer at pH 7.5.

HPr(Ser) Kinase Assay. Frozen cells of *M. capricolum* and *M. genitalium* were used to prepare crude extracts as described for the glycerol kinase assay. Incubations, in a volume of 10 μL, contained 20 mM Tris·HCl, pH 7.2, 5 mM NaF, 2 mM DTT, 2 mM $MgCl_2$, 5 mM fructose bisphosphate, 5 μg of *M. capricolum* HPr, 0.5 mM [γ-^{32}P]-ATP (∼1000 cpm/pmol), and the designated crude extract. Incubation was carried out for 30 min and terminated by the addition of SDS gel running buffer and then processed for gel electrophoresis as described in the legend to Figure 4.

RESULTS AND DISCUSSION

Constructs. The analysis of EIIA and the study of its interaction with HPr from *M. capricolum* necessitated the preparation of large amounts of highly purified proteins. To accomplish this, constructs capable of hyperexpression of EIIA and HPr were designed. The vector pMC-IIA for expression of *M. capricolum* EIIA was constructed as described under Materials and Methods.

For some of the experiments involving attempts to detect an HPr-EIIA interaction, it was necessary to use preparations of HPr of higher purity than generally obtained with the previously described pMC-HPr-based method (*22*). Consequently, a new derivative of pREI, designated pRE-His-Tag, was designed to allow the expression of proteins containing an N-terminal His tag (see Materials and Methods). This vector was then modified by insertion of the gene encoding *M. capricolum* HPr. The produced vector was designated pMC-His-Tag-HPr. The advantage of the expressed His-tagged HPr is that it was possible to purify this protein to near-homogeneity by nickel column affinity chromatography (see Materials and Methods).

Expression and Purification of M. capricolum Enzyme IIA^{glc}. pMC-IIA was transformed into *E. coli* host strain GI698, which encodes the λ repressor under control of the *trp* promoter (*23*). Maximum accumulation of EIIA was observed 4 h after the addition of tryptophan.

Washed cells were disrupted in a French press (2 passes, 10 000 psi) (Table 1). The crude extract was centrifuged at 100000*g* for 90 min. The supernatant solution after ultracentrifugation was fractionated by DE-52 chromatography (2.6 × 30 cm column and a salt gradient from 500 mL of 25 mM Tris·HCl, pH 7.5, to 500 mL of 25 mM Tris·HCl, pH 7.5 containing 300 mM NaCl). Fractions were analyzed by SDS−PAGE; those fractions enriched in EIIA were pooled and concentrated (using a dialysis bag, 3500 MW cutoff, packed in PEG). That material was then fractionated by Ultrogel AcA-44 gel filtration (2.6 × 100 cm column equilibrated with 25 mM Tris·HCl, pH 7.5, containing 0.1 M NaCl) and the sample was eluted with the same buffer. The pooled fractions from AcA-44 chromatography were mixed with an equal volume of Tris·HCl, pH 7.5, containing 2 M ammonium sulfate. The precipitate was dissolved in 25 mM Tris·HCl, pH 7.5, and the sample was fractionated on phenyl-Superose (HR10/10, Pharmacia) using an ammonium sulfate gradient from 1 to 0 M in 10 mM Tris·HCl, pH 7.5. The phenyl-Superose pool was then purified by FPLC Mono Q (HR 10/10) chromatography. Enzyme IIA^{glc} was eluted using a gradient from 3 to 10 mM NaCl in 50 mM Tris·HCl, pH 7.5. The final step in the purification involved FPLC Superose (12 HR 10/30) chromatography. The running buffer was 50 mM Tris·HCl, pH 7.5, containing 0.1 M NaCl. The final product was approximately 90% pure.

Table 1: Purification of Enzyme IIA^{glc} from *M. capricolum*[a]

purification step	total protein (mg)	purity (%)	enzyme IIA^{glc} (mg)
crude extract	7300	7.6	555
100000*g* supernatant	6100	7.6	464
DE-52 chromatography	624	16.8	105
AcA-44 chromatography	164	51.1	84
phenyl-Superose chromatography	112	66.2	74
Mono Q chromatography	92	85.7	78
Superose chromatography	68	90.3	61

[a] The details of the purification procedure are described under Results and Discussion.

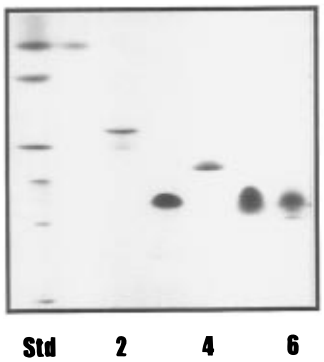

FIGURE 1: Isoelectric focusing analysis of enzymes IIA^{glc} from various sources. Purified preparations of the proteins and reference standards were electrophoresed on an isoelectric focusing gel with a pH range of 3−10. Electrophoresis was carried out at 100 V for 1 h, then 200 V for 1 h, followed by 300 V for 30 min. After equilibrium had been reached, the gel was fixed in 50% ethanol and 12.5% TCA, followed by washing with 25% ethanol and 8% acetic acid for 30 min. The gel was then stained with Coomassie blue R250. Std, protein standards (the bands, from top to bottom, correspond to proteins with p*I*s of 7.35, 6.55, 5.85, 5.2, and 4.55); lane 1, equine myoglobin, p*I* 7.3; lane 2, conalbumin, p*I* 5.9; lane 3, bovine serum albumin, p*I* 4.7; lane 4, *M. capricolum* EIIA; lane 5, *B. subtilis* EIIA; lane 6, *E. coli* EIIA.

Characterization of Purified Enzyme IIA^{glc}. Computer analysis of the presumptive translation product of the *M. capricolum crr* gene suggested that EIIA of *M. capricolum* has a higher p*I* (5.74) than the EIIA from *B. subtilis* (4.40) or *E. coli* (4.56) (*4*). A direct examination by IEF of the p*I*s of the proteins (Figure 1) showed that *M. capricolum*

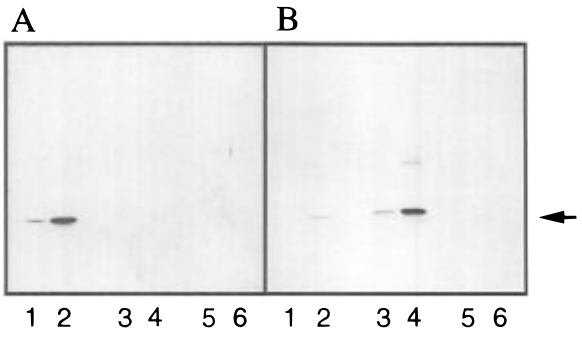

FIGURE 2: Western analysis of enzymes IIA^{glc}. Antisera against *E. coli* and *B. subtilis* EIIA were prepared by injecting purified proteins into rabbits. Proteins were electrophoresed on 4−20% Tris·glycine gradient PAGE gels (Novex minigels) and then transferred at 4 mA/cm^2 for 30 min onto immobilization membranes (Problott, Applied Biosystems) using an electroblotter (Sartoblot IIS, Sartorius). The procedure for transfer and immunostaining was as previously described (*4*). Lanes 1 and 2, 0.01 and 0.1 μg of *E. coli* EIIA, respectively; lanes 3 and 4, 0.01 and 0.1 μg of *B. subtilis* EIIA, respectively; lanes 5 and 6, 0.01 and 0.1 μg of *M. capricolum* EIIA, respectively. Antiserum against *E. coli* EIIA (panel A) or *B. subtilis* EIIA (panel B) was used for the Western blotting. The arrow corresponds to the position on the gels of EIIA.

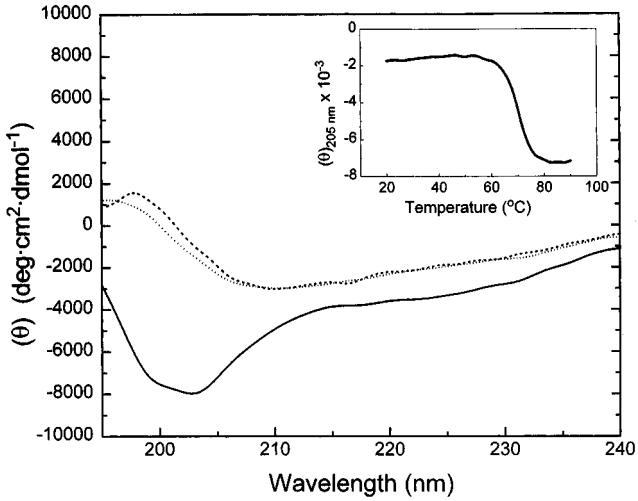

FIGURE 3: CD spectrum of *M. capricolum* enzyme IIA^{glc}. The CD spectrum was determined in 10 mM K·PO$_4$/1 mM DTT at pH 7.5 (0.36 mg/mL; 0.05 cm light path) at 20 °C (dashed line), at 90 °C (solid curve), and after equilibration for 1 h at 20 °C after heating to 80 °C (dotted curve). The inset shows temperature-induced changes in ellipticity at 205 nm from 20 to 90 °C using a heating rate of 60 °C/h.

EIIA focuses at a point characteristic of a higher p*I* (lane 4) than do the EIIAs from *B. subtilis* (lane 5) or *E. coli* (lane 6).

We previously showed (*4*) that *M. capricolum* EIIA could be phosphorylated by both Gram-negative and Gram-positive HPrs. Tests of phosphoacceptor activity (see Materials and Methods) indicated that the purified recombinant EIIA was a good acceptor of a phosphoryl group from P-HPr from *E. coli* (data not shown).

Tests of the immunological relatedness of *M. capricolum* EIIA to the homologous enzymes from *B. subtilis* and *E. coli* (Figure 2) showed that antiserum directed against *B. subtilis* EIIA was effective in detecting *B. subtilis* EIIA and poorly detected *E. coli* EIIA. The antiserum directed against *E. coli* EIIA was specific for the interaction with *E. coli* EIIA. Neither of the antisera was capable of interacting with *M. capricolum* EIIA. This was unexpected, since the *M. capricolum* EIIA shows significant phylogenetic similarity to the EIIA from *B. subtilis* (*4*) and can serve as a phosphoacceptor from *E. coli* HPr.

The far-UV circular dichroism spectrum of EIIA at 20 °C (Figure 3) is small in magnitude and typical for proteins containing β-sheet with little or no α-helical structures. However, the CD spectrum of EIIA has a minimum at ~208 nm rather than at 218 nm observed for β-sheet structures. This shift has been attributed to short lengths of β-strands separated by random coil loops (*24*). Secondary structural analysis for EIIA gave 11% α-helix, 20% turns, 47% β-sheet, and 21% random coil. These results suggest that *M. capricolum* EIIA adopts a structure similar to that of EIIAs from *B. subtilis* and *E. coli* (*25*).

CD spectra of EIIA are shown for 20 and 90 °C, and after cooling from 80 to 20 °C for 1 h (Figure 3). The refolded protein has approximately the same CD spectrum as that observed before heating to 80 °C; cooling from 90 °C gave a less complete return to the native spectrum (data not shown). The inset of Figure 3 shows the ellipticity decrease at 205 nm as a function of increasing temperature. Temperature increases at 30, 60, and 90 °C/h gave overlapping progress curves for thermal unfolding, and thermodynamic analysis (*18*) gave excellent fits to a two-state model with $T_m = 70 \pm 0.3$ °C and a van't Hoff ΔH of ~90 kcal/mol. Thus, EIIA (0.36 mg/mL) is quite stable and only undergoes reversible, two-state unfolding from 60 to 80 °C.

Table 2 summarizes analytical ultracentrifugation results obtained for EIIA (in the absence and presence of HPr), HPr, and enzyme I from *M. capricolum*. Enzyme IIA^{glc} from *M. capricolum* is the only EIIA described thus far that contains a cysteine residue. Whereas EIIA is monomeric in the presence of thiol reagent at either 4 or 20 °C, it was observed to form nonequilibrium mixtures of higher aggregates during an ultracentrifuge experiment in the absence of a reducing agent. HPr is monomeric with a molecular weight of 9400. Enzyme I from *M. capricolum* associates to dimers and tetramers at either 4 or 20 °C. At the concentrations of 4.3 $\times 10^{-6}$, 7.6 $\times 10^{-6}$, and 13.8 $\times 10^{-6}$ M enzyme I used in Table 2, the measured dimerization constant of 3.7 $\times 10^5$ (M monomer)$^{-1}$ (at 4 °C) indicates that approximately 50% dimer and only a small fraction of tetramer is present. Nevertheless, a satisfactory global fit of the sedimentation equilibrium data for enzyme I only could be obtained by assuming a monomer ↔ dimer ↔ tetramer equilibrium.

Previously, Cirillo and co-workers (*26*) reported that *M. capricolum* enzyme I was tetrameric in solution, composed of nonidentical subunits; we have presented evidence (*4*) that this is not the case. Kukuruzinska et al. (*27*) showed that enzyme I from *E. coli* undergoes a large temperature-dependent increase in the monomer−dimer association constant, increasing 280-fold between 8 °C and 23 °C; this did not occur with enzyme I from *M. capricolum*.

Interaction of Enzyme IIA^{glc} and HPr. Since the EIIA from *M. capricolum* is unique in that it contains a tryptophan residue (at position 2), a fluorescence-based approach was used to test for a complex by gel-filtration chromatography. No significant shift in the elution position of EIIA occurred after EIIA was mixed with HPr (both at 150 μM; data not shown).

If the HPr interaction with EIIA involved Trp-2, it might be expected that a fluorescence quenching would be observed. Titration of HPr up to a 26-fold molar excess into

Table 2: Sedimentation Equilibrium Studies on *M. capricolum* PTS Proteins (pH 7.5)[a]

protein	T (°C)	model for best fit	monomer M_r[b]	final SSQ (DOF)[c]
enzyme IIA^{glc}	20.0	nonassociation (monomer)	16 703	0.0070 (426)
enzyme IIA^{glc} + HPr[d]	20.0	nonassociation	16 700[d]	0.0084 (459)
HPr	20.0	nonassociation (monomer)	9 418	0.0051 (277)
enzyme I		association (monomer ↔ dimer ↔ tetramer)	64 602	
	4.0	dimerization log $K_A = 5.6$		0.0042 (267)
	20.0	dimerization log $K_A = 5.1$		0.013 (362)

[a] See Materials and Methods for ultracentrifugation conditions. [b] Monomer M_r values (calculated from amino acid compositions deduced from DNA sequences) were constrained during global, nonlinear least-squares fitting procedures. In all cases, the fitted concentration gradients ($\delta A/\delta R$) gave random distributions of residuals which were $\leq \pm 0.01$ absorbance unit. [c] Weighted sum of squared residuals (χ^2) and degree of freedom (DOF), where the degree of freedom for each fit is the number of data points minus the number of fitted parameters. [d] HPr was present in 2-fold molar excess of enzyme IIA^{glc}; radial scans were performed at 280 nm, where HPr does not absorb light.

Table 3: Effect of *M. capricolum* Enzyme IIA^{glc} on Glycerol Kinase Activity[a]

	% of control activity	
EIIA (mg/mL)	$-Zn^{2+}$	$+Zn^{2+}$
0.4	81	95
0.8	100	106
1.6	78	94

[a] Glycerol kinase activity was measured as described under Materials and Methods in a 100-μL volume containing 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2.5 mM dithiothreitol, 5 mM ATP, and 0.5 mM [^{14}C]glycerol (1000 cpm/pmol). *M. capricolum* crude extract (0.45 mg of protein/mL) was used as a source of glycerol kinase. The indicated amount of purified *M. capricolum* EIIA was added to incubation mixtures, some of which were supplemented with 1 mM $ZnCl_2$.

a solution of EIIA resulted in no change in the fluorescence (data not shown). The absence of any change might be due to the presence of the fluorescent residue in a region of the protein distal from the site of interaction with HPr. The model proposed by Herzberg (*28*) for the interaction of EIIA with HPr does not involve the amino-terminal region of EIIA in the complex.

Sedimentation equilibrium studies of EIIA in the presence of a 2-fold excess of HPr gave the same molecular weight for EIIA as observed in the absence of HPr (Table 2). Moreover, the addition of a 1.5-fold molar excess of HPr to EIIA at 30 °C in an isothermal titration calorimeter yielded no heat of reaction.

HPr was immobilized to a sensor chip in a BIAcore instrument. Sensorgrams were made of potential interaction of the HPr with EIIA in either the unphosphorylated or phosphorylated form. No interaction was observed (data not shown).

The conclusion from these five types of experiments designed to measure the interaction of EIIA with HPr is that the binding affinity is too low to measure by these methods.

Evaluation of Regulatory Properties of M. capricolum Enzyme IIA^{glc}. *E. coli* glycerol kinase is inhibited by *E. coli* EIIA; this inhibition is significantly enhanced by Zn^{2+} (*29*). The effect of EIIA on glycerol kinase from *M. capricolum* (Table 3) was tested at several concentrations of EIIA in the absence and presence of Zn^{2+}. The results showed no significant inhibitory effect of EIIA on the kinase activity.

Demonstration of HPr(Ser) Kinase Activity in M. capricolum. In the absence of any demonstrated regulation by EIIA, it seemed appropriate to search for an HPr(Ser) kinase activity in extracts of *M. capricolum* (Figure 4). In Gram-positive bacteria, HPr is phosphorylated on His-15 by a PEP-dependent HPr kinase (i.e., EI) or on Ser-46 by an ATP-dependent HPr kinase (*30*) that is allosterically regulated by fructose 1,6-bisphosphate (FBP) (*5*). Although HPrs of

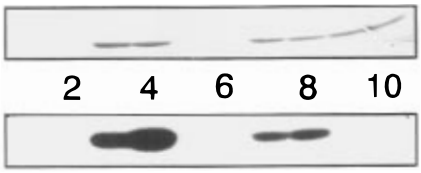

FIGURE 4: HPr(Ser) kinase activity in extracts of *M. capricolum* and *M. genitalium*. HPr(Ser) kinase activity was determined as described in Materials and Methods. The HPr substrate was derived from *M. capricolum*. After incubation, reaction mixtures were mixed with gel running buffer and deposited on SDS−polyacrylamide gels (4−20%, Tris·glycine, Novex). After electrophoresis, the gels were soaked in boiling TCA (15%) for 45 min and then stained with Coomassie Blue. Phosphorylated HPr was identified by autoradiography. Upper panel, stained gel; lower panel, autoradiogram. Lanes 1−4, extract from *M. capricolum* (0.05 μg/10 μL); lanes 5−8, extract from *M. genitalium* (0.5 μg/10 μL); lanes 9 and 10, extract from *M. genitalium* (0.05 μg/10 μL). Lanes 1 and 5, HPr and FBP omitted; lanes 2 and 6, HPr omitted; lanes 3, 7, and 9, FBP omitted; lanes 4, 8, and 10, complete reaction mixtures.

Gram-negative bacteria contain Ser-46, they are not phosphorylated by the ATP-dependent kinase. Evidence has been presented which suggests that HPr(Ser) phosphorylation may play a role in the regulation of various metabolic processes (*31*). The data presented in Figure 4 indicate that *M. capricolum* HPr is a substrate for FBP-activated ATP-dependent kinases present in both *M. capricolum* and *M. genitalium* extracts (see lanes 3 and 4, *M. capricolum*, and lanes 7 and 8, *M. genitalium*). However, the kinase activity was lower in *M. genitalium* extracts than in *M. capricolum* extracts (compare lanes 3 and 4 with lanes 9 and 10). Since *M. genitalium* HPr was not available, we could not determine whether differences in activity were due to differences in substrate specificity between the two kinases or whether the kinase is actually present in decreased amounts in *M. genitalium* extracts. It should be noted that *Acholeplasma laidlawii*, a species closely related to *M. capricolum*, lacks an intact PTS. While the organism lacks detectable enzyme II activities, it possesses HPr(Ser)kinase (*32*). It is also interesting to note that a gene encoding a putative serine/threonine kinase was identified in the genomic analysis of the *M. genitalium* genome (labeled MG109) (*2*). This kinase may be the protein responsible for the phosphorylation of HPr at Ser-47. The amino acid sequence in the vicinity of Ser-47 (Ser-46 in other Gram-positive bacteria) in *M. genitalium* is NIKSIINLM; this differs somewhat from the suggested signature sequence for ATP-dependent phosphorylation of HPr (NLKSIMXVM). The apparently lower activity of the *M. genitalium* kinase compared to the *M. capricolum* kinase in the studies presented above may be a reflection of these differences. These analyses argue against the possibility that EIIA serves the dual role of being a PTS

phosphocarrier as well as a regulator of sugar transporters and/or adenylyl cyclase, since the regulatory function might be adequately served by the HPr(Ser) phosphorylation mechanism.

It has been suggested (*33*) that Gram-positive bacteria do not possess the cytoplasmic EIIA protein and the regulatory mechanisms demonstrated in *E. coli* are lacking. Instead, these organisms possess an ATP-dependent metabolite-activated protein kinase that phosphorylates a seryl residue in HPr. Therefore, *M. capricolum* may be unique in having both the cytoplasmic EIIA and the HPr(Ser) kinase.

In summary, regulation of sugar transport in *M. capricolum* probably is exercised in the same way as in the typical Gram-positive bacteria. This is consistent with the suggestion that mycoplasmas were evolutionarily derived from Gram-positive bacteria.

Mapping of the Chromosomal Location of Genes Encoding PTS Proteins of M. capricolum. Previous sequencing studies (*3*, *4*) of *pts* genes in *M. capricolum* revealed an unusual arrangement. In *E. coli* or *B. subtilis* (*5*), the genes encoding HPr and enzyme I are located adjacent to one another. In contrast, the *M. capricolum* genes encoding these proteins were found to be separated on the chromosome. The recent report (*2*) of the sequence of the *M. genitalium* genome (580 kb) showed the *ptsH* and *ptsI* genes to be divergently expressed; the 3′-ends of the genes were separated by approximately 485 kb. Mapping studies, to see if the organization of these genes in the *M. capricolum* genome (1155 kb) was similar, in terms of spacing, to that of *M. genitalium*, were undertaken.

[^{32}P] probes for the *M. capricolum ptsH* and *ptsI* genes (see Materials and Methods) were used for the localization, by hybridization, of the positions of these genes on the *M. capricolum* chromosome (see Figure 5). The size distribution of the fragments generated by the different ENases has previously been described (*14*).

The data for the *ptsH* operon is shown in panels A (stained gel) and B (autoradiogram). The analysis shows hybridization (see Figure 5) to the *Sal*I fragment 1 (lane 1), the *Xho*I fragment 2 (lane 2), the *Mlu*I fragment 1 (lane 3), the *Apa*I fragment 1 (lane 4), the *Kpn*I fragment 2 (lane 5), the *Bgl*I fragment 1 (lane 7) and the *Bam*HI fragment 5 (lane 8). From these data, the deduced localization of the *ptsH* operon is in the region of the *Bam*HI fragment 5 that overlaps the *Kpn*I fragment 2 (see Figure 6).

The data for the *ptsI*−*crr* operon are shown in Figure 5, panels C (stained gel) and D (autoradiogram). The analysis shows hybridization to the *Sal*I fragment 1 (lane 1), the *Xho*I fragment 1 (lane 2), the *Mlu*I fragment 1 (lane 3), the *Apa*I fragment 1 (lane 4), the *Kpn*I fragment 3 (lane 5), the *Bgl*I fragment 3 (lane 7) and the *Bam*HI fragment 1 (lane 8). From these data, the deduced localization of the *ptsI*−*crr* operon is in the region of the *Bgl*I fragment 3 that overlaps the *Kpn*I fragment 3 (see Figure 6).

The assignment of the *ptsH* and *ptsI*−*crr* operons to the designated positions on the *M. capricolum* map is supported by computer analysis of the sequences previously reported for the *ptsH* (*3*) and *ptsI*−*crr* (*4*) operons. No recognition sites for any of the ENases used for the construction of the map were found in either of the operons (data not shown).

Mycoplasmas possess the smallest genomes of self-replicating bacteria (*34*). Since these organisms appear to utilize the minimum number of genes for growth, it might be predicted that most genes would be arranged on the chromosome to require the least amount of genetic information. It was therefore surprising to find that, in *M. capricolum* and *M. genitalium* (see Figure 7), the *ptsH* and *ptsI* operons are separated since this arrangement dictates a

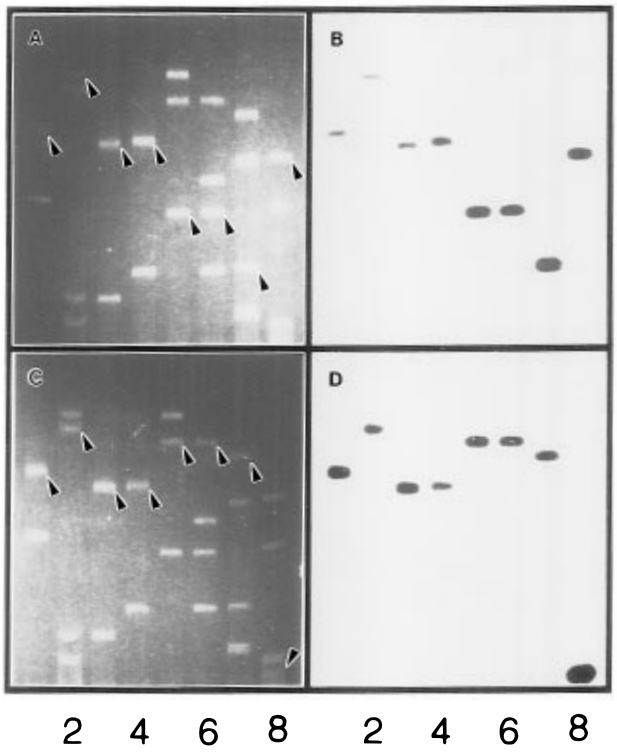

FIGURE 5: Localization of *ptsH* and *ptsI*−*crr* operons on the chromosome of *M. capricolum*. Chromosomal DNA of *M. capricolum* was digested with a variety of ENases and then subjected to FIGE, and Southern hybridization was performed with probes (see Materials and Methods, Southern blotting) for the *ptsH* and *ptsI*−*crr* operons. Panels A and C, stained gel; panel B, autoradiogram of membrane probed for *ptsH*; panel D, autoradiogram of membrane probed for *ptsI*. Lane 1, DNA digested with *Sal*I; lane 2, DNA digested with *Sal*I + *Xho*I; lane 3, DNA digested with *Mlu*I; lane 4, DNA digested with *Apa*I; lane 5, DNA digested with *Kpn*I; lane 6, DNA digested with *Apa*I + *Kpn*I; lane 7, DNA digested with *Bgl*I; lane 8, DNA digested with *Bam*HI. The bands in the agarose gel corresponding to those producing positive hybridization signals in panels B and D are indicated by arrowheads in panels A and C.

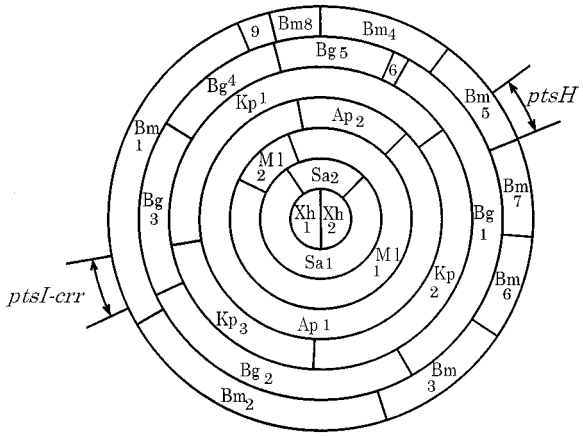

FIGURE 6: Location of *ptsH* and *ptsI*−*crr* operons on the physical map of *M. capricolum*. Fragments generated by digestion with ENases are arranged in their respective orders on the circular chromosome (1156 kb) of *M. capricolum* (*14*). The sizes of the indicated fragments have previously been described (*14*). Bm, *Bam*HI; Bg, *Bgl*I; Kp, *Kpn*I; Ap, *Apa*I; Ml, *Mlu*I; Sa, *Sal*I; Xh, *Xho*I. The positions of the *ptsH* and *ptsI*−*crr* operons deduced from the hybridization data of Figure 5 are indicated by the bracketed arrows.

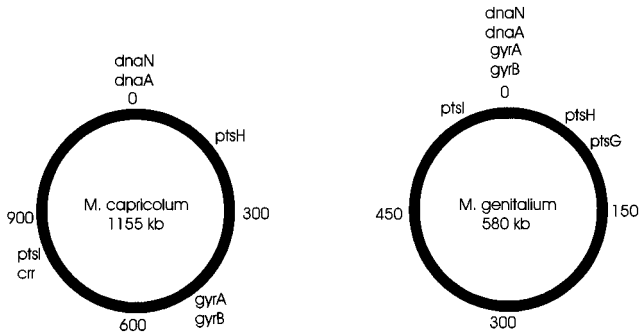

FIGURE 7: Comparison of location of genes on the maps of *M. capricolum* and *M. genitalium.*

requirement for distinct promoters (*3*, *4*; this work). This is in marked contrast to the organization of the genes encoding the PTS proteins enzyme I and HPr in other organisms (*5*).

Mycoplasmas are believed to have evolved from Gram-positive bacteria with a 2200−2500-kb genome by the selective elimination of nonessential genes (*35*). On the basis of the observations presented here showing the probable change from a fused *pts* operon to two operons that are separated by about 500 kb on the genome (see Figure 7), it seems likely that part of the evolutionary process involved some type of recombinational event. The finding that genes encoding DNA gyrase are located antipodally to the *dnaA*−*dnaN* region in the *M. capricolum* chromosome (*36*) but in the same region of the chromosome in eubacteria (*37*) and in *M. genitalium* lends further support to the idea that many recombinations occurred in the evolution of mycoplasmas.

Concluding Remarks. In this paper, we have described the properties of purified EIIA from *M. capricolum*. The unique presence in this EIIA of a tryptophan residue was taken advantage of to analyze the protein using a fluorescence assay. The unique presence in this EIIA of a cysteine residue was demonstrated to introduce the possibility for dimer formation. In contrast to the corresponding proteins from *E. coli*, *M. capricolum* EIIA shows little interaction with HPr. While EIIA from *E. coli* regulates the activity of glycerol kinase, no such activity regulation is observed in *M. capricolum*.

In the course of this work, a new generally applicable vector for expressing His-tagged proteins was constructed.

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REFERENCES

1. Sharp, P. M., and Li, W.-H. (1987) *Nucleic Acids Res. 15*, 1281−1295.
2. Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., Bult, C. J., Kerlavage, A. R., Sutton, G., Kelley, J. M., et al. (1995) *Science 270*, 397−403.
3. Zhu, P.-P., Reizer, J., Reizer, A., and Peterkofsky, A. (1993) *J. Biol. Chem. 268*, 26531−26540.
4. Zhu, P.-P., Reizer, J., and Peterkofsky, A. (1994) *Protein Sci. 3*, 2115−2128.
5. Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993) *Microbiol. Rev. 57*, 543−594.
6. Reizer, J., and Peterkofsky, A. (1987) in *Sugar Transport and Metabolism in Gram-positive Bacteria* (Reizer, J., and Peterkofsky, A., Eds.) pp 333−364, Ellis Horwood, Chichester, U.K.
7. Reizer, J., Sutrina, S. L., Saier, M. H., Jr., Stewart, G. C., Peterkofsky, A., and Reddy, P. (1989) *EMBO J. 8*, 2111−2120.
8. Seok, Y.-J., Lee, B. R., Zhu, P.-P., and Peterkofsky, A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 347−351.
9. Reddy, P., Peterkofsky, A., and McKenney, K. (1989) *Nucleic Acids Res. 17*, 10473−10488.
10. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
11. Shinomiya, T., and Ina, S. (1991) *Nucleic Acids Res. 19*, 3935−3941.
12. Koob, M., and Szybalski, W. (1992) *Methods Enzymol. 216*, 13−20.
13. Miyata, M., Sano, K.-I., Okada, R., and Fukumura, T. (1993) *Nucleic Acids Res. 21*, 4816−4823.
14. Miyata, M., Wang, L., and Fukumura, T. (1991) *FEMS Microbiol. Lett. 79*, 329−334.
15. Waddell, W. J. (1956) *J. Lab. Clin. Med. 48*, 311−314.
16. Laemmli, U. K. (1970) *Nature 227*, 680−685.
17. Yang, J. T., Wu, C. S., and Martinez, H. M. (1986) *Methods Enzymol. 130*, 208.
18. Kirchhoff, W. T. (1993) NIST Technical Note 1401, U.S. Government Printing Office, Washington, DC.
19. Zamyannin, A. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 145−165.
20. Zolkiewski, M., and Ginsburg, A. (1992) *Biochemistry 31*, 11991−12000.
21. Novotny, M. J., Frederickson, W. L., Waygood, E. B., and Saier, M. H., Jr. (1985) *J. Bacteriol. 162*, 810−816.
22. Zhu, P.-P., Lecchi, P., Pannell, L., Jaffe, H., and Peterkofsky, A. (1995) *Protein Expression Purif. 6*, 189−195.
23. LaVallie, E. R., DiBlasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F., and McCoy, J. M. (1993) *Bio/Technology 11*, 187−193.
24. Garcia, K. C., de Sauvage, F. J., Struble, M., Henzel, W., Reilly, D., and Goeddel, D. V. (1993) *J. Biol. Chem. 268*, 22397−22401.
25. Herzberg, O., and Klevit, R. (1994) *Curr. Opin. Struct. Biol. 4*, 814−822.
26. Ullah, A. H. J., and Cirillo, V. P. (1977) *J. Bacteriol. 131*, 988−996.
27. Kukuruzinska, M. A., Turner, B. W., Ackers, G. K., and Roseman, S. (1984) *J. Biol. Chem. 259*, 11679−11681.
28. Herzberg, O. (1992) *J. Biol. Chem. 267*, 24819−24823.
29. Feese, M., Pettigrew, D. W., Meadow, N. D., Roseman, S., and Remington, S. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3544−3548.
30. Reizer, J., Novotny, M. J., Hengstenberg, W., and Saier, M. H., Jr. (1984) *J. Bacteriol. 160*, 333−340.
31. Reizer, J., Romano, A. H., and Deutscher, J. (1993) *J. Cell. Biochem. 51*, 19−24.
32. Hoischen, C., Dijkstra, A., Rottem, S., Reizer, J., and Saier, M. H., Jr. (1993) *J. Bacteriol. 175*, 6599−6604.
33. Saier, M. H., Jr., Chauvaux, S., Deutscher, J., Reizer, J., and Ye, J. J. (1995) *Trends Biochem. Sci. 20*, 267−271.
34. Razin, S. (1985) *Microbiol. Rev. 49*, 419−455.
35. Maniloff, J. (1992) in *Mycoplasmas; Molecular Biology and Pathogenesis* (Maniloff, J., McElhaney, R. N., Finch, L. R., and Baseman, J. B., Eds.) pp 549−559, American Society for Microbiology, Washington, DC.
36. Sano, K.-I., and Miyata, M. (1994) *Gene 151*, 181−183.
37. Yoshikawa, H., and Ogasawara, N. (1991) *Mol. Microbiol. 5*, 2589−2597.

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