Enzyme I of the Phosphoenolpyruvate:Sugar Phosphotransferase System Has Two Sites of Phosphorylation per Dimer†

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ABSTRACT: Enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system of *Escherichia coli* has been reported to contain one phosphorylation site per dimer and thus operates by either a half of the sites or a flip-flop mechanism [Misset, O., & Robillard, G. T. (1982) *Biochemistry 21*, 3136–3142; Hoving, T., ten Hoeve-Duurkens, R., & Robillard, G. T. (1984) *Biochemistry 23*, 4335–4340]. In this paper, the determination of two phosphorylation sites per dimer of enzyme I was made by using a number of different methods. In some experiments, less than two sites per dimer were found, but a concomitant loss in enzyme I activity was also found. The phosphorylated residue in enzyme I was shown to have the properties expected for a N^3-phosphohistidinyl residue.

The phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] was first discovered in *Escherichia coli* by Kundig et al. (1964). The PTS is responsible for the concomitant phosphorylation and translocation across the cell membrane of a number of hexoses and hexitols. It appears that the phosphorylation of any of these substrates requires five phosphoryl transfer steps from phosphoenolpyruvate and, depending upon the hexose or hexitol, three or four proteins and enzymes are involved (Saier et al., 1985). The one common component to all these sugar-specific systems is enzyme I which is a protein kinase in which a phosphoenzyme intermediate form exists (Waygood & Steeves, 1980; Hoving et al., 1981). Closely related to the *E. coli* PTS is the PTS found in *Salmonella typhimurium*; the proteins and enzymes of the two systems have been used interchangeably and have been found, when studied in detail, to be either identical or very similar. However, some dissimilarities have been reported particularly with respect to enzyme I.

The subunit molecular weight of the *E. coli* enzyme I as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was reported to be 84 000 (Robillard et al., 1979). Subsequent reports have stated that the subunit molecular weight is about 70 000 ± 2000 and that the dimer is M_r 134 000 (Misset et al., 1980). Waygood and Steeves (1980) reported a subunit molecular weight of 67 000 ± 5000 and a dimer molecular weight of 135 000. Roseman and co-workers (Weigel et al., 1982a; Kukuruzinska et al., 1982) have shown that the *S. typhimurium* enzyme I has a subunit molecular weight of 64 000 ± 4000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and 57 700 by sedimentation equilibrium studies. The *S. typhimurium* enzyme I also forms dimers. The mobility of enzyme I from *E. coli* and *S. typhimurium* on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels is identical and is usually the same as bovine serum albumin (Waygood et al., 1984). The p*I* values for enzyme I from both species appear to be identical (Waygood & Mattoo, 1983a; Mattoo et al., 1984), and amino acid analyses of the enzymes are very similar (Waygood & Steeves, 1980; Weigel et al., 1982a). Antibodies raised against the *E. coli* enzyme I show a high degree of cross-reactivity with *S. typhimurium* enzyme I, but some differences between the two enzymes were detected (Mattoo & Waygood, 1983a).

The kinetic parameters of enzyme I from the two species were reported to be identical and to show typical properties of a Bi-Bi ping-pong mechanism (Waygood & Steeves, 1980; Weigel et al., 1982a). Robillard and co-workers have reported that the kinetics of the *E. coli* enzyme I do not conform to a Bi-Bi ping-pong mechanism and that only one phosphorylation site exists per dimer. They conclude that enzyme I has a half of the sites reactivity mechanism or a flip-flop mechanism (Misset & Robillard, 1982; Hoving et al., 1984). Enzyme I from *S. typhimurium* was shown to have two phosphorylation sites per dimer, and phosphorylation resulted in the formation of a 3-phosphohistidinyl residue (Weigel et al., 1982b). Enzyme I from *E. coli* is presumed to contain a 3-phosphohistidinyl residue as it has the same stability as the phosphoryl residue found in *S. typhimurium* enzyme I (Waygood et al., 1984).

This fundamental difference in the number of phosphorylation sites between two such closely related enzymes is difficult to rationalize, and in this paper, we report the characterization of phosphorylated enzyme I from *E. coli* and conclude that this enzyme is similar to the *S. typhimurium* enzyme I in that there are two phosphorylation sites per dimer.

Materials and Methods

Materials. Phosphoenolpyruvate was synthesized as described previously by Clark and Kirby (1963). [^{32}P]-Phosphoenolpyruvate was synthesized as previously described (Mattoo & Waygood, 1983b). DEAE-cellulose paper (DE81) and DEAE-cellulose (DE32) were obtained from Whatman. Lactate dehydrogenase (beef heart, type III), pyruvate kinase (rabbit muscle), dithiothreitol, dithioerythritol, ϵ-aminocaproic acid, adenosine 5′-diphosphate, NADH, Hepes, Mes, and bovine serum albumin were obtained from Sigma. Phosphoenol[1-^{14}C]pyruvate and 2-[U-^{14}C]deoxyglucose were obtained from Amersham. [U-^{14}C]Glucose was obtained from Schwarz/Mann.

Protein Preparations. Enzyme I and HPr were prepared from *E. coli* P650 as has been described (Waygood & Steeves,

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[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system; HPr, histidine-containing phosphocarrier protein of the PTS; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DTE, dithioerythritol; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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1980). The HPr preparations usually contained some HPr-1 which is of no consequence (Waygood et al., 1985). ϵ-Aminocaproic acid (40 mM), a protease inhibitor, was incorporated in the buffers during purification of enzyme I (Mattoo & Waygood, 1983a). The preparation of enzyme I was incubated with 5 mM phosphoenolpyruvate and 5 mM $MgCl_2$ and was dialyzed against 0.01 M Hepes buffer, pH 7.5, with 0.2 mM dithioerythritol and 1 mM EDTA before use, except where noted.

Protein Measurement. Both enzyme I and HPr concentrations were measured by the biuret procedure (Layne, 1957); by the spectrophotometer method of Waddell (1956) where $OD_{215} - OD_{225} = 144\ \mu g\ mL^{-1}\ ODU^{-1}$. HPr concentration was determined by the following: the lactate dehydrogenase coupled assay (Waygood et al., 1979); measurement of ^{32}P incorporation by the DEAE-cellulose paper chromatography method (Anderson et al., 1971; Wiegel et al., 1982b; Waygood et al., 1985). All HPr determinations gave essentially the same value. Enzyme I concentration was determined by the following: $OD_{280} = 4.0$ for a 10 mg/mL solution of enzyme I. This was determined from a dry weight sample of enzyme I which had been dialyzed against water. Misset and Robillard (1982) reported an $\epsilon_{280nm}^{10mg} = 3.6$, and Waygood and Steeves (1980) reported a value of 4.4. The microbiuret procedure (Layne, 1958) gave consistently lower values for both enzyme I and HPr.

Molecular Weights Used To Determine Protein Concentration. The molecular weight of HPr (9120) is well established by sequencing (Powers & Roseman, 1985; De Reuse et al., 1985). The molecular weight of enzyme I is not as well established. Subunit molecular weights for enzyme I from both *E. coli* and *S. typhimurium* when determined by sodium dodecyl sulfate gel electrophoresis gave values that were consistently about 64000–67000 (Waygood & Staeeves, 1980; Weigel et al., 1982a; Waygood et al., 1984). More recently, the *S. typhimurium* enzyme I is reported to be M_r 60000 (Kukuruzinska et al., 1985). The extensive hydrodynamic studies gave M_r 57700 (Kukuruzinska et al., 1982). The initial reports from Robillard's group gave a subunit molecular weight of 84000 (Robillard et al., 1979), but this has subsequently modified downward to M_r 70000 (Misset et al., 1980). For the purpose of calculation of subunit concentration, a value of M_r 60000 was used. This figure is a convenient compromise between the values obtained by ultracentrifugation and electrophoresis.

Measurement of Phosphoenolpyruvate. Phosphoenolpyruvate was checked by assaying its conversion to pyruvate in the presence of ADP and pyruvate kinase using a coupled assay with lactate dehydrogenase and NADH. [^{32}P]-Phosphoenolpyruvate which contained at most 2% [^{32}P]P_i was checked by following its conversion to pyruvate and [α-^{32}P]-ATP, as previously described (Mattoo & Waygood, 1983b). Phosphoenol[1-^{14}C]pyruvate was checked by following its conversion to [1-^{14}C]pyruvate by pyruvate kinase and ADP and by their separation by DEAE-cellulose paper chromatography as described by Mattoo and Waygood (1983b). Phosphoenol[1-^{14}C]pyruvate contained 15–20% ^{14}C label that remained at the origin when chromatographed. This impurity could be removed by ion-exchange chromatography (Mattoo & Waygood, 1983b).

Determination of HPr Concentration by [^{32}P]Phospho-HPr Isolation. HPr (about 20 nmol) was incubated with 1 mM [^{32}P]phosphoenolpyruvate (specific activity 10000 cpm/nmol), 10 mM $MgCl_2$, 0.05 M Hepes buffer, pH 6.8, 0.2 nmol of enzyme I monomer, and 0.2 mM DTE in a total volume of 0.05 mL. Samples (0.02 mL) were taken after 5- and 10-min incubation at 37 °C. Each sample was added to an equal volume of 0.2 M sodium carbonate and mixed. Of this mixture, 0.03 mL was placed on a strip of DEAE-cellulose paper and chromatographed as described previously (Anderson et al., 1971). [^{32}P]Phospho-HPr remained at the origin while the [^{32}P]phosphoenolpyruvate and [^{32}P]P_i moved with the solvent front.

Isolation of [^{32}P]Phosphoenzyme I. Enzyme I (0.3 mL of a 5.6 mg/mL solution) was incubated at 37 °C for 60 min with 0.025 μmol of HPr, 1 mM [^{32}P]phosphoenolpyruvate (specific activity 20000 cpm/nmol), 5 mM $MgCl_2$, 50 mM Hepes buffer, pH 6.8, and 0.2 mM DTE in a 1-mL volume. Samples (0.02 mL) were taken to measure the extent of enzyme I phosphorylation. The incubation mixture was cooled and chromatographed on a 2.5 × 100 cm G50-Sephadex column equilibrated with 0.01 M potassium phosphate buffer, pH 8.0, containing 1 mM EDTA and 0.2 mM DTE. A similar incubation using 0.1 mM [^{32}P]phosphoenolpyruvate (specific activity 400000 cpm/nmol) was carried out for only 20 min and separated as above to give [^{32}P]phosphoenzyme I with higher specific activity for the hydrolysis experiments.

Production of Dephosphorylated Enzyme I. Enzyme I was incubated with an equimolar amount of HPr at 37 °C and pH 6.8. The hydrolysis rate of the 1-phosphohistidinyl (1-P-histidinyl) residue in P-HPr under these conditions gave a half-life of about 5 min (Waygood et al., 1985). Incubation was for 60 min for 50 nmol of enzyme I and HPr in 1 mL of 0.01 mM potassium phosphate buffer, pH 6.5, with 0.2 mM DTE and 1 mM EDTA. The incubation mixture was cooled on ice, diluted with 5 mL of 0.1 M potassium phosphate buffer, pH 7.5, with 0.2 mM DTE and 1 mM EDTA, and loaded onto a 1-mL DEAE-cellulose column (DE-23) equilibrated with the same buffer. HPr, which did not bind under these conditions, was completely eluted with 10 mL of the equilibration buffer; enzyme I was eluted by using 1 M potassium phosphate buffer, pH 6.5, with 0.2 mM DTE and 1 mM EDTA, and 0.5-mL fractions were collected. Phosphorylation measurements were performed on the fractions with the highest amount of protein present and on the same fractions following dialysis against 10 mM potassium phosphate buffer, pH 7.5, with 0.2 mM DTE and 1 mM EDTA. This procedure is similar to the method of Wiegel et al. (1982b).

Determination of [^{32}P]Phosphoenzyme I. Enzyme I was incubated with [^{32}P]phosphoenolpyruvate usually at 1 mM concentration containing 20000 cpm/nmol. Samples were taken with time, and the [^{32}P]phosphoenzyme I formed was determined by chromatography on DEAE-cellulose paper (Weigel et al., 1982b).

Enzyme I Activity. The activity of enzyme I was determined by using the general methods described by Waygood et al. (1979). Dilution of the enzyme from concentrated solutions (1–5 mg/mL) was carried out by the following procedure. Enzyme I was incubated for 10 min at 37 °C. The enzyme was then diluted 5-fold into 0.01 M Hepes buffer, pH 7.0, with 0.2 mM DTE, 1 mM EDTA, 5 mM phosphoenolpyruvate, and 10 mM $MgCl_2$. This was maintained at 37 °C for about 5 min. The enzyme was then finally diluted 500–2000-fold into the same warm buffer. Samples of these dilutions were assayed by using 10 mM [U-^{14}C]glucose or 2-[U-^{14}C]deoxyglucose (specific activity 100000 cpm/μmol, 30 μM HPr, and 10 mM phosphoenolpyruvate in 0.05-mL assays as previously described (Waygood et al., 1979; Waygood & Steeves, 1980). A unit of activity is the number of micromoles of sugar produced per minute.

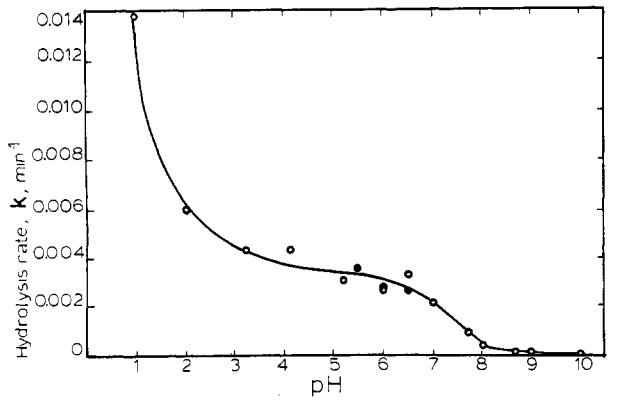

FIGURE 1: pH dependence of the hydrolysis rates of phosphoenzyme I at 37 °C. [^{32}P]Phosphoenzyme I was isolated by G-50 Sephadex chromatography and was incubated with buffers as described under Materials and Methods. The buffers were HCl–KCl, pH 1.1 and 2.0; citrate–phosphate, pH 3.2–6.5; phosphate, pH 7.0–8.6; bicarbonate, pH 9 and 10.0 [all (○)], or Tris–acetate, pH 5.5–6.5 (●).

Separation of [^{32}P]Phosphoenzyme I and [^{32}P]Phospho-HPr and [^{32}P]Phosphoenolpyruvate by HPLC. Samples of incubation mixtures containing [^{32}P]phosphoenzyme I, [^{32}P]P-HPr, and [^{32}P]phosphoenolpyruvate were separated by molecular sieve chromatography using a Beckman Altex HPLC. The separation was on a 3000SW TSK-Spherogel column (7.5 mm × 30 cm) supplied by Beckman. The column was equilibrated with 25 mM potassium phosphate, pH 8.0, buffer with 0.2 mM DTE, which was pumped at 1 mL/min. The 0.02-mL samples were imbedded in a 0.05-mL loading sample to ensure complete loading. The eluate was monitored at 215 nm with a Hitachi 100-40 spectrophotometer equiped with a flow cell. Fractions (1 mL) were collected directly into scintillation vials from 5- to 17-min elution time, and all fractions were counted for ^{32}P by using 5 mL of Beckman Ready-Solv EP scintillation fluid. Enzyme I eluted between 7 and 9 min with a peak at 7.9 ± 0.1 min. HPr eluted completely between 11 and 12 min, with a peak at 11.4 ± 0.1 min. [^{32}P]Phosphoenolpyruvate eluted at between 12 and 14 min with a peak at 12.6 ± 0.1 min. Although [^{32}P]-phosphoenolpyruvate and [^{32}P]P-HPr eluted closely to each other, the separation was complete.

Determination of Phosphoenzyme I by the Pyruvate Burst Method. Enzyme I was mixed with 0.01 M Hepes buffer, pH 7.5, with 1 mM EDTA, 0.2 mM DTE, 5 mM $MgCl_2$, 0.15 mM NADH, and 50 μg of lactate dehydrogenase in a total volume of 1 mL. A base-line rate for NADH oxidation was established after about 5 min. Phosphorylation of enzyme I was initiated by the addition of 0.005 mL of 0.2 M phosphoenolpyruvate, and the resulting pyruvate formation was measured by the amount of NADH oxidation and was followed at 340 nm with a Gilford recording spectrophotometer. Experiments were carried out at room temperature.

pH Dependence of the Hydrolysis Rates of Phosphoenzyme I. [^{32}P]Phosphoenzyme I was isolated by G-50 Sephadex chromatography as described above and was incubated with buffers at 37 °C such that their concentration was 25 mM and a 0.05-mL sample contained about 4000 cpm. At appropriate times, the samples were added to 0.05 mL of ice-cold 0.2 M sodium carbonate, and this mixture was applied to, and chromatographed on, DEAE-cellulose paper to separate ^{32}P-phosphoprotein and [^{32}P]P_i as described previously (Wiegel et al., 1982b; Waygood et al., 1985). The buffers are described in the figure legends. The hydrolysis rate (k) values were calculated from the results from six to nine time points for each pH value.

Table I: Hydrolysis of the 3-Phosphohistidinyl Residue in Phosphoenzyme I[a]

added component	concn (M)	k_{obsd}	k_1	k_2[b]
buffer	0.025	0.0024	0.0024	
pyridine	0.05	0.022		0.39
hydroxylamine	0.20	0.048		0.023
L-histidine	0.05	0.0043		0.039
	0.10	0.0056		0.032
	0.20	0.0060		0.022

[a] The buffer was 0.05 M Mes, pH 6.5, and compounds added were prepared in this buffer and adjusted to pH 6.5. Equal volumes of [^{32}P]phosphoenzyme I and additives were mixed to initiate hydrolysis. [b] $k_2 = (k_{obsd} - k_1)$/concn of added compound.

Table II: Hydrolysis of the 3-Phosphohistidinyl Residue in Phosphoenzyme I in the Presence of $MgCl_2$

buffer	[$MgCl_2$] (mM)	k_{obsd}	k_1	k_2[a]
0.05 M Mes, pH 6.1	0	0.0033	0.0033	
	2.5	0.0041		0.33
	10	0.0056		0.23
0.05 M Hepes, pH 7.5	0	0.0019	0.0019	
	2.5	0.0040		0.85
	10	0.0069		0.50

[a] $k_2 = (k_{obsd} - k_1)$/concn of added compound.

RESULTS

Properties of the Phosphorylated Residue in E. coli Enzyme I. [^{32}P]Phosphoenzyme I was isolated and incubated with buffers at 37 °C to determine the pH dependence of the rate of hydrolysis of the phosphoamino acid residues. The results are shown in Figure 1 and were typical of the behavior of a 3-P-histidinyl residue (Hultquist et al., 1966). There was no deviation from normal behavior as has been found for the 1-P-histidinyl residue in P-HPr (Waygood et al., 1985). The effects of various nucleophilic agents on the hydrolysis rate are given in Table I and are also typical for a 3-P-histidinyl residue. The results in Table II show that the hydrolysis rate was stimulated by $MgCl_2$ addition. The hydrolysis rate of the 3-P-histidinyl residue in enzyme I was investigated at pH 6.5 and pH 8.0 by using sterile preparations kept on ice and at 3 ± 0.5 °C. The half-lives at pH 6.5 were 25 days at 3 °C and 30 days in ice. At pH 8.0, the hydrolysis rates were considerably slower, being at least twice as long as the pH 6.5 values.

Number of Phosphorylation Sites. All the experimental approaches reported in this paper started with the assumption that there was an unknown but considerable amount of phosphorylation in the enzyme I preparation and that this must be removed before a reliable measurement could be made. This was because the enzyme I purification was carried out with phosphorylated enzyme I. A number of experiments all of which were based upon the same general principles and rationale were carried out. If enzyme I was fully phosphorylated when purified, then incubation with HPr and [^{32}P]-phosphoenolpyruvate would result in the transfer and exchange of the unlabeled phosphate. If the [^{32}P]phosphoenolpyruvate was in excess, then eventually all the unlabeled phosphate would be diluted either by exchange reactions or by hydrolysis of the 1-P-histidinyl residue in P-HPr. If enzyme I were incubated with HPr in the absence of phosphoenolpyruvate, then the phosphoryl group in enzyme I would be transferred to HPr to form P-HPr. At pH 6.5 and 37 °C, the half-life of the 1-P-histidinyl residue in P-HPr is about 5 min (Waygood et al., 1985); thus, a 3-h incubation with about equimolar amounts of enzyme I and HPr would result in essentially the

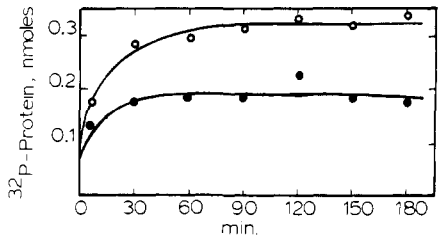

FIGURE 2: Phosphorylation of enzyme I. Enzyme I, HPr, and 1 mM [^{32}P]phosphoenolpyruvate were incubated together at 37 °C, and samples (0.02 mL) were taken at the times shown, loaded onto an HPLC molecular sieve column, and eluted as described under Materials and Methods. The samples contained by protein estimation 0.33 nmol of enzyme I and 0.27 nmol of HPr. The figure shows the nanomoles of ^{32}P-phosphoprotein that eluted at the enzyme I position (○) and the HPr position (●).

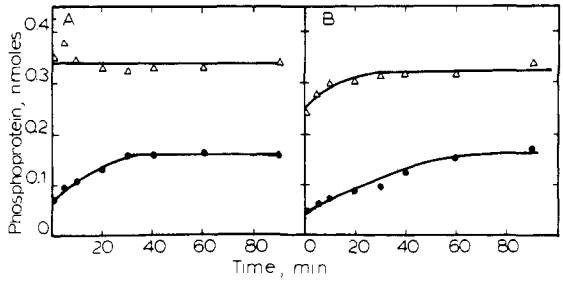

FIGURE 3: Phosphorylation of enzyme I. A preparation of enzyme I was dialyzed against (A) Hepes buffer as described under Materials and Methods or (B) 25 mM sodium phosphate buffer, pH 7.0, with 1 mM dithiothreitol as used by Misset and Robillard (1982). Both were then incubated with 1 mM [^{32}P]phosphoenolpyruvate with HPr (△) and without HPr (●). Samples (0.02 mL) were taken at the times shown and applied to DEAE-cellulose paper, and the total phosphoprotein was determined as described under Materials and Methods. The samples contained 0.18 nmol of enzyme I and 0.16 nmol of HPr by protein estimation. The figure shows the total phosphoprotein formed in the 0.02-mL sample, where (△) represents the combined values of phosphoenzyme I and P-HPr.

complete hydrolysis of all the phosphate in the two proteins, to produce dephosphorylated enzyme I.

Various combinations of enzyme I, HPr, and [^{32}P]-phosphoenolpyruvate were used to estimate the amount of phosphorylated in enzyme I. The three experiments described (Figures 2–4) were all carried out by using incubations at 37 °C with 1 mM [^{32}P]phosphoenolpyruvate (specific activity 25 000 cpm/nmol) and about 0.008–0.02 mM HPr and enzyme I. The specific details are in the figure legends. The samples applied to either the DEAE-cellulose paper chromatograms or the HPLC columns were 0.02-mL aliquots of such incubations.

Figure 2 shows the data for an incubation that lasted 180 min. Samples were applied to a HPLC molecular sieve column which separated [^{32}P]phosphoenzyme I, [^{32}P]phospho-HPr, and [^{32}P]phosphoenolpyruvate. The results show that the isolated enzyme I had one phosphorylation site per subunit or two per dimer. The HPLC column chromatography was run such that [^{32}P]P-HPr eluted in about 11 min, which, at pH 8.0 and room temperature, would result in about 20–25% hydrolysis of the 1-P-histidinyl residue in P-HPr (Waygood et al., 1985). The [^{32}P]P-HPr recovered was therefore equivalent to 100% phosphorylation when the sample was applied to the column.

Robillard's group used enzyme I dialyzed against 25 mM sodium phosphate buffer, pH 7.0, with 1 mM dithiothreitol. This different buffer did not influence the degree of phosphorylation as the results in Figure 3 show. In this, enzyme I dialyzed against the two different buffers gave essentially

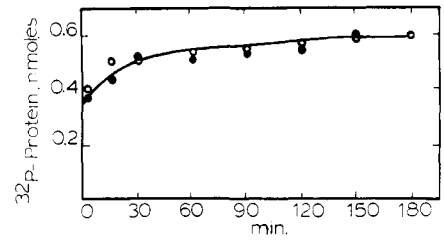

FIGURE 4: Phosphorylation of enzyme I after incubation with HPr. Enzyme I and HPr were incubated under two conditions. The first condition was identical with that in Figure 2 except that the samples contained 0.26 nmol of HPr and 0.34 nmol of enzyme I by protein estimation (○). The second condition involved an incubation of enzyme I and HPr together but without [^{32}P]phosphoenolpyruvate. For this second condition, at the time points shown, samples were taken and then incubated with 1 mM [^{32}P]phosphoenolpyruvate for 1 min. From these incubations, samples were taken containing amounts of P-HPr and phosphoenzyme I identical with the samples described for the first condition (●). The ^{32}P-phosphoprotein in both samples was separated by DEAE-cellulose paper chromatography.

the same results. In addition, a slower exchange between phosphoenzyme I and [^{32}P]phosphoenolpyruvate occurred in the absence of HPr and gave essentially one phosphorylation site per monomer. The apparent differences in the rate of phosphorylation (Figure 3A compared to Figure 3B) were not observed in other experiments.

The third type of experiment (Figure 4) involved the incubation of enzyme I and HPr together without any phosphoenolpyruvate. Samples were taken at the appropriate times and then incubated with 1 mM [^{32}P]phosphoenolpyruvate for 1 min. Following this, appropriate samples were applied to the DEAE-cellulose paper chromatographs. Similar HPLC experiments were carried out (results not shown). The results were again consistent with one site per subunit for enzyme I.

Lastly, [^{32}P]phosphoenzyme I isolated from the G50-Sephadex column as described under Materials and Methods had 0.8 phosphorylation site per monomer.

Isolation of Dephosphorylated Enzyme I. To cause dephosphorylation, enzyme I was incubated with HPr, and separation of HPr and enzyme I was accomplished by DEAE-cellulose chromatography as described under Materials and Methods. This preparation could be directly phosphorylated by [^{32}P]phosphoenolpyruvate, and a pyruvate burst was measured by coupling the enzyme I phosphorylation with lactate dehydrogenase. Two observations were made. The first was that by either [^{32}P]phosphoenolpyruvate labeling or spectrophotometric assay for the pyruvate burst, 0.5–0.6 site of phosphorylation per monomer was measured. This results agreed with the results reported by Robillard's group, but as described below, the specific activity of enzyme I was greatly reduced. The second observation was that a phosphoenolpyruvate phosphatase was detected by the spectrophotometric assay used for the pyruvate burst method. The activity at room temperature was 7 nmol min^{-1} (mg of enzyme I)$^{-1}$ which is more than 100 times less than the phosphorylation activity of enzyme I. As this activity is greater than the spontaneous hydrolysis rates shown in Figure 1 and in Table II, it seems probable that this is not an intrinsic phosphatase activity which is associated with many phosphoryl transfer enzymes (Knowles, 1980) but rather a contaminating phosphatase activity.

Specific Activity vs. Phosphorylation. When the enzyme I preparations used for the experiments in Figures 2–4 had been stored on ice for periods in excess of 7–10 days, a decline in the ability of the preparations to be phosphorylated occurred. Experiments similar to the ones described in Figures 2–4 produced results which gave a variety of results down to 0.5 site per monomer. However, when the preparations were

assayed for activity by using saturating concentrations of HPr and phosphoenolpyruvate, the specific activity of the preparations had declined from 200 ± 20 units/mg at 37 °C to 100 ± 20 units/mg. The isolated dephosphorylated enzyme I used for the pyruvate burst experiments had a specific activity of 90 units/mg. The reasons for this decline in activity are unknown, but enzyme I is usually stored in the presence of phosphoenolpyruvate and $MgCl_2$ to stabilize it (Waygood & Steeves, 1980). The preparations used in these experiments were stored in the absence of these two ligands. In all cases, there was a direct correlation between specific activity and the degree of phosphorylation.

Discussion

Enzyme I from *S. typhimurium* has been shown by Weigel et al. (1982b) to have two phosphohistidinyl residues per dimer. Enzyme I from *E. coli*, which is very similar as described in the introduction, has been found to have only one such phosphorylation per dimer (Misset & Robillard, 1982; Hoving et al., 1984). In contrast, the results presented in this paper show that *E. coli* enzyme I has two sites of phosphorylation per dimer. Enzyme I was shown to have two sites per dimer when HPr was included with enzyme I to facilitate the removal of the preexisting phosphoryl group in enzyme I and also when a slow exchange with [^{32}P]phosphoenolpyruvate occurred in the absence of HPr (Figure 3). Identical results were obtained when enzyme I was incubated with HPr in the presence of [^{32}P]phosphoenolpyruvate which would ensure continued phosphorylation of enzyme I, or when [^{32}P]phosphoenolpyruvate was introduced after the dephosphorylation of enzyme I by HPr (Figure 4). While the DEAE-cellulose paper chromatography used for most experiments (e.g., Figures 3 and 4) involved the separation of both phosphoproteins together (i.e., P-HPr and phosphoenzyme I), the HPLC separation gave an independent measurement of phosphoenzyme I and the same result of two phosphorylation sites per dimer (Figure 2).

It is stated by Hoving et al. (1984) that they have used the DEAE-cellulose paper chromatography method and have found only one phosphorylation site per dimer of enzyme I. In addition, Misset and Robillard (1982) state that the result is the same whether phosphorylation is measured in the presence or absence of HPr, using the pyruvate burst method of Brouwer et al. (1982). Hoving et al. (1984) report one phosphorylation site per dimer as determined by the DEAE-cellulose paper chromatography method. This is the principle method used in this paper and also for the determination of two phosphorylation sites per dimer for enzyme I of *S. typhimurium* (Wiegel et al., 1982).

Attempts were made to repeat the experiments reported by Robillard's group. Unfortunately, in none of the three papers that report the one site per dimer (Brouwer et al., 1982; Misset & Robillard, 1982; Hoving et al., 1984) is there a description about the amount of enzyme I used or the concentration of phosphoenolpyruvate used, except for a brief description of the conditions used for the soluble factor of the PTS of *Rhodopseudomonas sphaeroides* (Brouwer et al., 1982). This laboratory has been unable to achieve sufficient separation between pyruvate and phosphoenolpyruvate by the methods described by Hoving et al. (1981) in order to use the pyruvate burst method described by Brouwer et al. (1982). In attempting to carry out the pyruvate burst method by coupling the reaction to lactate dehydrogenase, a method that requires milligram quantities of dephosphorylated enzyme I, a lower amount of phosphorylation was found but was correlated with a concomitant loss in activity. This approach was not pursued because the phosphoenolpyruvate phosphatase activity associated with the enzyme I preparation was of a sufficient magnitude to give uncertainty as to how much of the measured change in absorbance was due to the pyruvate burst or due to the phosphatase activity. It should be noted that Brouwer et al. (1982) report a hydrolysis activity for the soluble factor of the PTS of *R. sphaeroides*. Hoving et al. (1982) report a hydrolysis activity for phosphoenzyme I with a rate constant of 0.05 min^{-1} in the presence of 2.5 mM $MgCl_2$ at pH 7.2 at an unspecificed temperature. This rate constant is considerably higher than that reported in Figure 1 for isolated [^{32}P]-phosphoenzyme I hydrolysis, and 2.5 mM $MgCl_2$ gave only a small activation of the rate constant (Table II). The phosphatase activity measured by the lactate dehydrogenase coupled assay was 7 nmol min^{-1} (mg of enzyme I)$^{-1}$ which, according to the assumptions of Brouwer et al. (1982), corresponds to a hydrolysis rate of 0.45 min^{-1} (assuming two phosphorylation sites per dimer). It would therefore appear that both enzyme I preparations were contaminated with a phosphatase.

While carrying out the experiments reported in this paper, less than two sites per dimer and even one site per dimer have been observed. These lower values have always been associated with a concomitant loss in the specific activity of enzyme I. According to the assay methods described here, enzyme I has a specific activity equivalent to 200 μmol of sugar phosphate produced per minute per milligram at 37 °C. The specific activity reported by Robillard's group initially was the same value (Robillard et al., 1979), but when a modified purification procedure was used, the specific activity decreased to 100 μmol min^{-1} (mg of enzyme I)$^{-1}$ (Misset & Robillard, 1982). The assay system used for the two different preparations of enzyme I from Robillard's group was the same; however, Misset and Robillard (1982) contend that the difference was due to the vagaries of the assay system and not due to differences in the enzyme I preparation.

There are significant differences in the purification procedures between our laboratory and Robillard's group. While the strain used is the same, *E. coli* P650, there are differences in the purification steps and the solutions in which enzyme I is maintained (Robillard et al., 1979; Waygood & Steeves, 1980; Misset & Robillard, 1982; Mattoo & Waygood, 1983). The stability of enzyme I in dilute solutions is poor, and it has been shown that phosphorylation has a major stabilizing effect. Moreover, repeated incubations of enzyme I with phosphoenolpyruvate and $MgCl_2$ during purification result in greatly improved yields of active enzyme I (Waygood & Steeves, 1980). In addition, we have found that enzyme I even when substantially pure is susceptible to proteolytic degradation during purification which results in a loss in specific activity and the inability to form a sharply focused band on native isoelectric focusing gels (unpublished results). Thus, ε-aminocaproic acid, a protease inhibitor, was included in all buffers (except during ionic exchange chromatography) to which enzyme I is exposed (Mattoo & Waygood, 1983). Neither of these precautions, which we have found necessary to produce highly active enzyme I, has been employed by Robillard's group.

One phosphorylation site per dimer of enzyme I is consistent with the kinetic mechanism for *E. coli* enzyme I as proposed by Misset and Robillard (1982). The different kinetic mechanism (i.e., simple Bi-Bi ping-pong) which has been reported by Waygood and Steeves (1980) for *E. coli* enzyme I, and Wiegel et al. (1982) for *S. typhimurium* enzyme I, is said to be theoretically incorrect because the influence of the monomer–dimer equilibrium of enzyme I was neglected

(Misset & Robillard, 1982). The two opposing views of how enzyme I behaves kinetically are derived from experiments carried out under substantially different conditions. Misset and Robillard (1982) dilute enzyme I in cold buffer before carrying out assays. Such a procedure is known to cause the dissociation of enzyme I ino its subunits (Waygood et al., 1979; Waygood & Steeves, 1980; Misset et al., 1980; Hoving et al., 1982). The introduction of enzyme I cold and dilute does produce a kinetic response that is dependent upon both enzyme I concentration and substrate concentration. The influence of the monomer–dimer equilibrium can be eliminated by the preincubation conditions described by Waygood et al. (1979), Waygood and Steeves (1980), and Weigel et al. (1982) in which enzyme I is kept warm and phosphorylated before dilution into assays. This observation has also been made by Misset et al. (1980). We would suggest that the physiological reality of an enteric bacteria is such that internal protein concentrations, internal substrate concentrations, and normal growth temperatures favor the view that dissociation of enzyme I into monomers is not a significant event prior to phosphorylation. It has been shown by Misset and Robillard (1982) that for enzyme I at concentrations below physiological levels, the influence of metal ion and protein concentration is sufficient to give dimerization and that phosphorylation furthers dimer formation at even lower protein concentrations. The report of Kukuruzinska et al. (1985), in which dissociation of *S. typhimurium* enzyme I following phosphorylation is shown, does not contradict the view that enzyme I must be dimerized to become phosphorylated. However, Hoving et al. (1982) find that phosphorylation stabilizes the dimer, and that is also our experience. Recently, evidence for trimer formation has been presented (Grenier et al., 1985).

Clearly, there are a number of unresolved issues about the properties of enzyme I and its mechanism of action. With respect to the number of phosphorylation sites in *E. coli* enzyme I, it is necessary to have an unambiguous measurement of its specific activity after purification by the method of Misset and Robillard (1982). The evidence in this paper suggests that the correlation between specific activity and the number of phosphorylation sites is strong. There seems to be general agreement that the dimer form is required for enzyme I phosphorylation, but the question now raised by Kukuruzinska et al. (1985) is whether phosphorylation promotes dissociation into subunits and whether this is a required step for HPr phosphorylation. It seems very unlikely given all the similarities between the PTS in *E. coli* and *S. typhimurium* that differences in mechanism and catalysis can be satisfactorily explained on the basis of species difference.

The results in this paper show that enzyme I can be phosphorylated on both its subunits at the same time which favors the view that enzyme I has a simple ping-pong Bi-Bi kinetic mechanism.

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