Phosphoenolpyruvate-Dependent Protein Kinase Enzyme I of *Streptococcus faecalis*: Purification and Properties of the Enzyme and Characterization of Its Active Center†

C.-A. Alpert,‡ R. Frank,§ K. Stüber,‖ J. Deutscher,‡ and W. Hengstenberg*,‡

Department of Microbiology, Ruhr-Universität Bochum, NDEF 06, D-4630 Bochum, West Germany, European Molecular Biology Laboratories, D-6900 Heidelberg, West Germany, and Institute for Genetics, University of Köln, D-5000 Köln, West Germany

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ABSTRACT: Enzyme I, the phosphoenolpyruvate:protein phosphotransferase (EC 2.7.3.9), which is part of the bacterial phosphoenolpyruvate- (PEP) dependent phosphotransferase system, has been purified from *Streptococcus faecalis* by using a large-scale preparation. Size exclusion chromatography revealed a molecular weight of 140 000. On sodium dodecyl sulfate gels, enzyme I gave one band with a molecular weight of 70 000, indicating that enzyme I consists of two identical subunits. The first 59 amino acids of the amino-terminal part of the protein have been sequenced. It showed some similarities with enzyme I of *Salmonella typhimurium*. The active center of enzyme I has also been determined. After phosphorylation with [^{32}P]PEP, the enzyme was cleaved by using different proteases. Labeled peptides were isolated by high-performance liquid chromatography on a reversed-phase column. The amino acid composition or amino acid sequence of the peptides has been determined. The largest labeled peptide was obtained with Lys-C protease and had the following sequence: -Ala-Phe-Val-Thr-Asp-Ile-Gly-Gly-Arg-Thr-Ser-His*-Ser-Ala-Ile-Met-Ala-Arg-Ser-Leu-Glu-Ile-Pro-Ala-Ile-Val-Gly-Thr-Lys-. It has previously been shown that the phosphoryl group is bound to the N-3 position of a histidyl residue in phosphorylated enzyme I. The single His in position 12 of the above peptide must therefore carry the phosphoryl group.

The phosphoenolpyruvate (PEP)[1]-dependent protein kinase enzyme I is part of the PEP-dependent phosphotransferase system (PTS) which catalyzes carbohydrate uptake in microorganisms (Hengstenberg, 1977; Dills et al., 1980; Robillard, 1982).

The PTS is described by the following reaction scheme:

$$\text{PEP} + \text{enzyme I} \overset{Mg^{2+}}{\rightleftharpoons} \text{P-enzyme I} + \text{pyruvate} \quad (1)$$

$$\text{P-enzyme I} + \text{HPr} \rightleftharpoons \text{P-HPr} + \text{enzyme I} \quad (2)$$

$$\text{P-HPr} + \text{sugar} \xrightarrow[\text{enzyme III, } Mg^{2+}]{\text{enzyme II}} \text{sugar-P} + \text{HPr} \quad (3)$$

Enzyme I has been shown to be phosphorylated by PEP at the N-3 position of a histidyl residue (Hengstenberg et al., 1976; Weigel et al., 1982b). As outlined in eq 2, P-enzyme I phosphorylates the phospho-carrier protein HPr, which then transfers the phosphoryl group to the sugar. The last reaction step usually requires a membrane component, enzyme II, which carries the sugar specificity and a soluble, enzyme II specific component which is called either enzyme III or factor III. Besides its well-known function as HPr kinase, enzyme I may be involved in the complex regulatory phenomenon of catabolite repression (Peterkofsky, 1975).

In this paper, we describe a large-scale purification method for enzyme I of *Streptococcus faecalis* which allowed further characterization of this protein. As both enzyme I and factor III proteins interact with P-HPr, we expected to find similarities in the active centers of these proteins. Like enzyme I, factor III proteins have been shown to be phosphorylated by P-HPr at the N-3 position of a single histidyl residue (Kalbitzer et al., 1981; Dörschug et al., 1984).

Materials and Methods

Bacterial Strains. S. faecalis 26487 (Streptococcenzentrale Kiel) was grown in a 100-L Chemap fermenter at 37 °C. One hundred liters of medium contained 1000 g of yeast extract (Ohly, Hamburg), 200 g of tryptone (Difco), 200 g of Na_2HPO_4, and 1000 g of glucose. The pH was kept at 7 by titration with 10% NaOH. After 5–6 h of growth (OD_{578nm} was around 10), cells were harvested with a Westfalia continuous-flow centrifuge. The yield was 1000 g of wet cell paste.

Purification of Enzyme I. A 300-g sample of cell paste of *S. faecalis* 26487 was suspended in 600 mL of standard buffer (0.05 M Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM PMSF, and 0.1 mM DTT) and disrupted in a Dynomill KDL (Bachofen, Switzerland) as described by Beyreuther et al. (1977). Cell-free extract was obtained after centrifugation at 10000*g* for 2 h.

The cell-free extract was applied to a Whatman DE-23 column (12 × 20 cm), which was eluted with a 8-L linear gradient of 0–0.8 M NaCl in standard buffer. Fractions of 100 mL were collected with a Serva Linear II Makro fraction

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‡ Ruhr-Universität Bochum.

§ European Molecular Biology Laboratories.

‖ University of Köln.

[1] Abbreviations: PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate-dependent phosphotransferase system; HPr, histidine-containing phospho-carrier protein; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; TFA, trifluoroacetic acid; P-enzyme I, phosphorylated enzyme I; HPLC, high-performance liquid chromatography; Me_2SO, dimethyl sulfoxide; PEI, poly(ethylenimine); TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; P-Ser-HPr, HPr phosphorylated at a seryl residue.

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Table I: Purification Procedure of Enzyme I from *Streptococcus faecalis*[a]

	protein (mg)	activity (units)[b]	yield (%)	sp act. (units/mg)	purification factor
DE-23	12200	12.9×10^3	100	1.06	1
supernate of 50% $(NH_4)_2SO_4$ pptn[c]	4400				
pellet of 70% $(NH_4)_2SO_4$ pptn[c]	3700				
supernate, pH 5.1	2000	6.9×10^3	53	3.4	3.2
supernate, pH 4.7	1400	4.8×10^3	37	3.3	3.1
G-150	360	5.4×10^3	42	14.9	14
HA[d]	54	4.6×10^3	35	85	80

[a] Sometimes an additional run on a DE-52 column was necessary (refer to Materials and Methods). [b] One unit = 1 μmol of *o*-nitrophenyl β-D-galactopyranoside hydrolyzed in a mutant complementation assay in 10 min at 37 °C. [c] $(NH_4)_2SO_4$ influences the test. [d] Hydroxylapatite.

collector. Enzyme I containing fractions were localized with a mutant complementation assay (Hengstenberg et al., 1969), pooled, and precipitated with $(NH_4)_2SO_4$, 50–70% saturation. After centrifugation at 20000*g* for 30 min, the pellet was dissolved in standard buffer and dialyzed against 5 L of 50 mM sodium acetate buffer (pH 5.1) and subsequently against sodium acetate buffer at pH 4.7. The dialysate was centrifuged at 20000*g* for 30 min, and the supernate was applied to a G-150 Sephadex column (5 × 100 cm), eluted with standard buffer. Enzyme I containing fractions were loaded on an HA ultrogel column (5 × 20 cm) and eluted with a 2.4-L linear gradient of 1–200 mM sodium phosphate, pH 6. Enzyme I fractions were pooled and applied to a Whatman DE-52 column (1.5 × 20 cm) run with a linear gradient of 500 mL of 0–500 mM NaCl in standard buffer. Fractions with enzyme I activity were desalted on a Sephadex G-25 column (3 × 25 cm) by using 50 mM NH_4HCO_3, pH 8, as the volatile buffer. Enzyme I protein was lyophilized and stored at 4 °C.

Synthesis of [^{32}*P*]*PEP.* [^{32}P]PEP was synthesized by using a modified method of Lauppe et al. (1972): 0.1 mL (1 mCi) of [^{32}P]orthophosphate (carrier free) (Buchler) was evaporated in a vacuum centrifuge (Savant), 100 μL of a solution of 6.2 mg of β-chlorolactic acid (Sigma) dissolved in 1 mL of acetonitrile was added, and the solvent was evaporated in a vacuum centrifuge by using an oil pump and a cold trap. The azeotropic drying process was repeated twice with 100 μL of acetonitrile; 50 μL of Me_2SO, 5 μL of triethylamine, and 1 μL of trichloroacetonitrile were added. The solution turned yellow, and the reaction was stopped after incubation for 2 h at 37 °C by adding 20 μL of H_2O. The solvents acetonitrile, triethylamine, and dimethyl sulfoxide were dried over CaH_2, distilled, and stored over CaH_2. Trichloroacetonitrile was distilled before use. The reaction mixture was separated by chromatography on PEI–cellulose thin-layer plates (Macherey and Nagel) in the solvent system 0.3 M KH_2PO_4. ^{32}P-Labeled compounds were localized by autoradiography.

More conveniently, the reaction products can be separated by HPLC on a Servachrom Si 100 Polyol DEAE column (5 μm, 4.6 × 250 mm; Serva). The column was eluted with a linear gradient of 0–30% solvent B in 30 min: solvent A, 5 mM NaH_2PO_4; solvent B, 1 M NaH_2PO_4; flow rate, 1 mL/min; temperature, 40 °C.

The radioactivity was measured with a Berthold LB 504 HPLC radioactivity monitor (Berthold, Wildbad, West Germany). The flow cell contained a Teflon tube to measure Cerenkov radiation.

High-Performance Liquid Chromatography. For chromatography on a Nucleosil C 8 reversed-phase column (Macherey and Nagel), we used the following equipment: low-pressure gradient former M 250 B (Gynkothek, Munich), Jasco HPLC pump (Biotronik), Rheodyne 1725 syringe sample injector, and Jasco Uvidec 100 III UV monitor with variable wavelength.

For size-exclusion chromatography, we used either a G-2000 SW column (LKB) or a Biosil TSK 125 column (Bio-Rad). Both columns were run with a Model 396 Minipump (Milton Roy LDC) equipped with a pulse dampener (Labomatic) and a pressure gauge. The flow rates were from 0.3 to 1 mL/min.

Isolation of the Active Center Peptide. To isolate the active center of enzyme I, we incubated 1 mg of enzyme I (14 nmol), dissolved in 0.2 mL of 50 mM NH_4HCO_3 and 5 mM $MgCl_2$, pH 8, together with 0.1–0.5 μCi of [^{32}P]PEP (carrier free) for 10 min at 37 °C. Then 250 nmol of cold PEP was added, and the assay mixture was incubated for a further 5 min to achieve complete phosphorylation of enzyme I. The ^{32}P content of enzyme I was estimated after separation of [^{32}P]-PEP and ^{32}P-enzyme I on a TSK size-exclusion column. After completion of enzyme I phosphorylation, one of the following proteases was added to the reaction mixture: trypsin, TPCK treated (Worthington), or Lys-C or Arg-C protease (Boehringer, Mannheim). The ratio of protease to protein was 1:20, and the incubation time was 30 (trypsin) or 90 min (Lys-C or Arg-C protease). Peptides were separated on a Nucleosil C8 10-μm column (Macherey and Nagel, Düren) using an acetonitrile gradient: solvent A, 20 mM potassium phosphate, pH 6.8; solvent B, 80% acetonitrile, HPLC grade (Baker).

The column was eluted with a linear gradient from 0% to 60% solvent B in 60 min at a flow rate of 1 mL/min. Radioactive fractions were collected manually and applied to the same column, now equilibrated with 0.1% TFA. Chromatography was performed by using a linear gradient from 0% to 60% solvent B in 60 min (solvent A, 0.1% trifluoroacetic acid; solvent B, 85% acetonitrile). The radioactive fractions were evaporated in a vacuum centrifuge. Peptides isolated by this procedure were salt free and suitable for amino acid analysis.

Amino Acid Analysis. Salt-free peptides were hydrolyzed for 24 h in 6 N HCl at 110 °C and analyzed on a Biotronic amino acid analyzer. PTH-amino acids were analyzed by HPLC reversed-phase chromatography on a Zorbax-CN column (4 mm × 250 mm, Du Pont).

Sequence Determination. The amino acid sequence was determined by Edman degradation on a liquid phase sequence analyzer. The amino acid residues were identified by the HPLC technique. The active center peptide, obtained with Lys-C protease, was sequenced on a gas phase sequencer according to Hewick et al. (1981).

NaDodSO$_4$ Gels. NaDodSO$_4$ gels were prepared according to Weber & Osborn (1969) and contained 7.5% acrylamide.

Results

Enzyme I was purified from *S. faecalis* 26487 as described under Materials and Methods. Table I displays the purification scheme. It was advantageous to use acidic conditions during certain steps of the purification. In acidic solution, enzyme I is stable for several months at 4 °C. In contrast, we find that at pH 7 and higher, enzyme I loses its activity

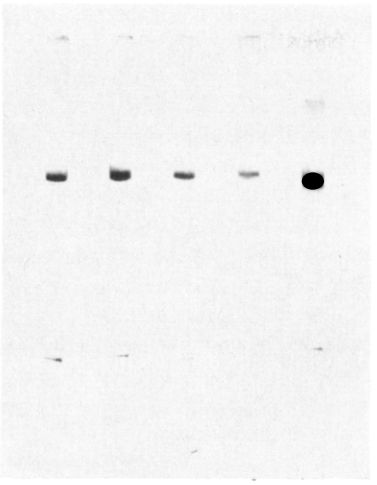

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified enzyme I. From left to right, the lanes contained 10, 20, 10, and 5 μg of enzyme I. The far right lane contains the marker protein bovine serum albumin (68 000 daltons, 20 μg).

rapidly. This is in agreement with reports about the stability of enzyme I from *Escherichia coli* or *Salmonella typhimurium* (Waygood & Steeves, 1980). The amount of enzyme I isolated from *S. faecalis* is similar to those reported by other groups (Waygood et al., 1980; Robillard et al., 1979; Weigel et al., 1982) who have purified enzyme I from Gram-negative organisms.

The protein was homogeneous according to native polyacrylamide gel electrophoresis, $NaDodSO_4$ gel electrophoresis, and isoelectric focusing which revealed a p*I* value of 4.2 for enzyme I. Figure 1 shows $NaDodSO_4$ gels of enzyme I and bovine serum albumin as marker protein. Enzyme I migrates according to a molecular weight of 70 000. The molecular weight of enzyme I, obtained by size-exclusion chromatography on a G-2000 SW column, was found to be 140 000. This indicates that enzyme I consists of two identical subunits. Dissociation of enzyme I into its subunits could not be observed, neither in the phosphorylated nor in the dephosphorylated state.

The high purity of the protein preparation allowed us to determine the following amino-terminal amino acid sequence by automated Edman degradation up to position 59, which is also a strong argument for the identity of the subunits: NH_2-Ser-Glu-Met-Leu-Lys-Gly-Ile-Ala-Ala-Ser-Asp-Gly-Val-Ala-Val-Ala-Lys-Ala-Tyr-Leu-Leu-Val-Gln-Pro-Asp-Leu-Ser-Phe-Asn-Lys-Thr-Ser-Val-Glu-Asp-Thr-?-Ala-Glu-Ala-Thr-?-Leu-Asp-His-Ala-Ser-Met-Lys-Met-Thr-?-Glu-Gln-Gly-?-Ile-?-Asp. The first 59 amino acid residues contain only one histidyl residue in position 45 as a possible phosphorylation site by PEP. A comparison of the N-terminal part of enzyme I of *S. faecalis* with the N-terminal part of enzyme I of *S. typhimurium* (Weigel et al., 1982) revealed some similarities when the first amino acid residue of the latter was shifted to the third residue of *S. faecalis* enzyme I. Out of 17 amino acids, 8 were found to be identical, and some positions showed only minor changes (Leu > Ile, Lys > Arg). The good correspondence is demonstrated by a "hydropathy" profile of the N-terminal part of the two proteins according to Kyte & Doolittle (1982) (Figure 2).

In further experiments, we tried to localize the active center of enzyme I by isolating a labeled peptide following phosphorylation of enzyme I with [^{32}P]PEP. So far, studies concerned with the characterization of the active center of enzyme I were restricted to the isolation of ^{32}P-labeled enzyme I. Larger proteolytic fragments including the active center have not yet been isolated from enzyme I due to methodical problems during the separation of very complex peptide mixtures obtained after proteolytic cleavage of a 70 000-dalton polypeptide chain. The rapid progress in peptide separation by

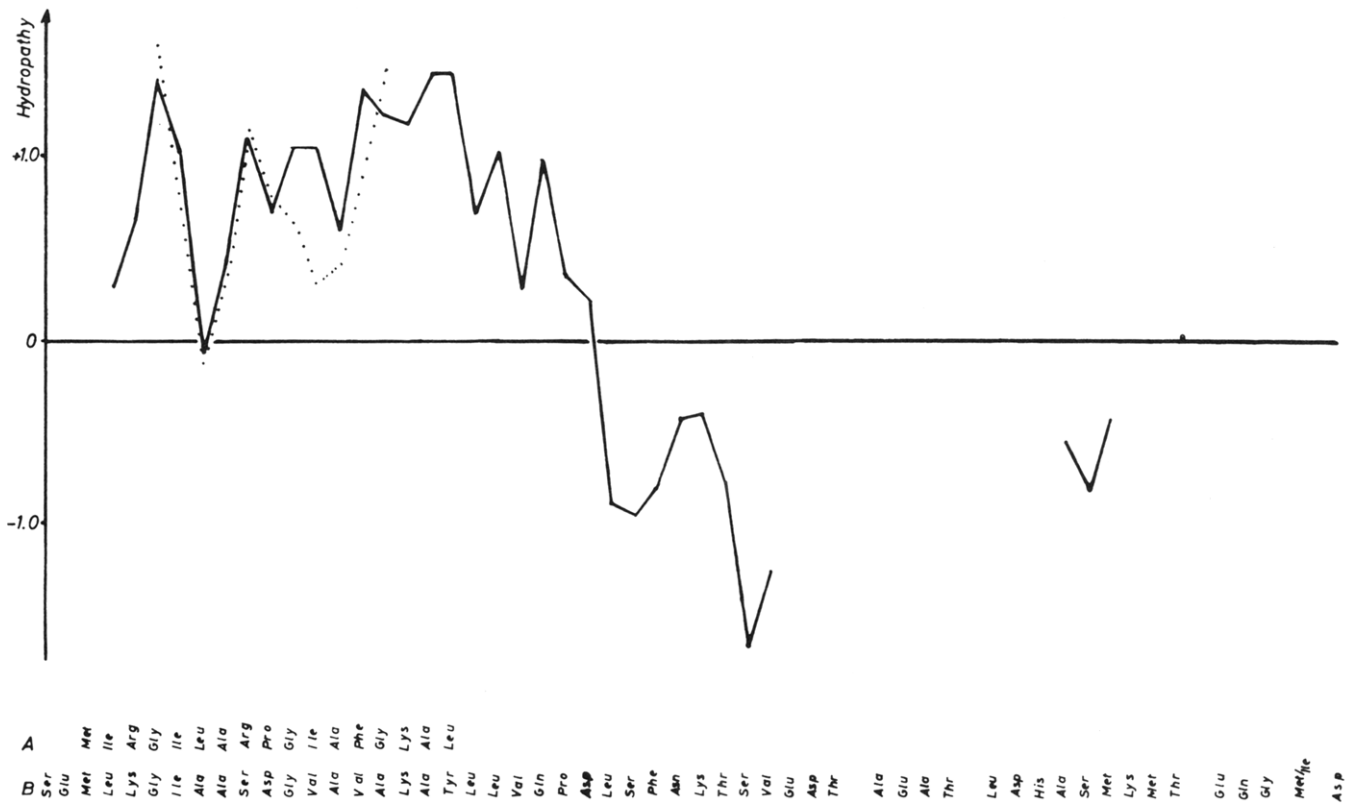

FIGURE 2: Comparison of the hydropathy of different enzyme I proteins according to Kyte & Doolittle (1982): (···) from *Salmonella typhimurium* (Weigel et al., 1982a); (—) from *Streptococcus faecalis*.

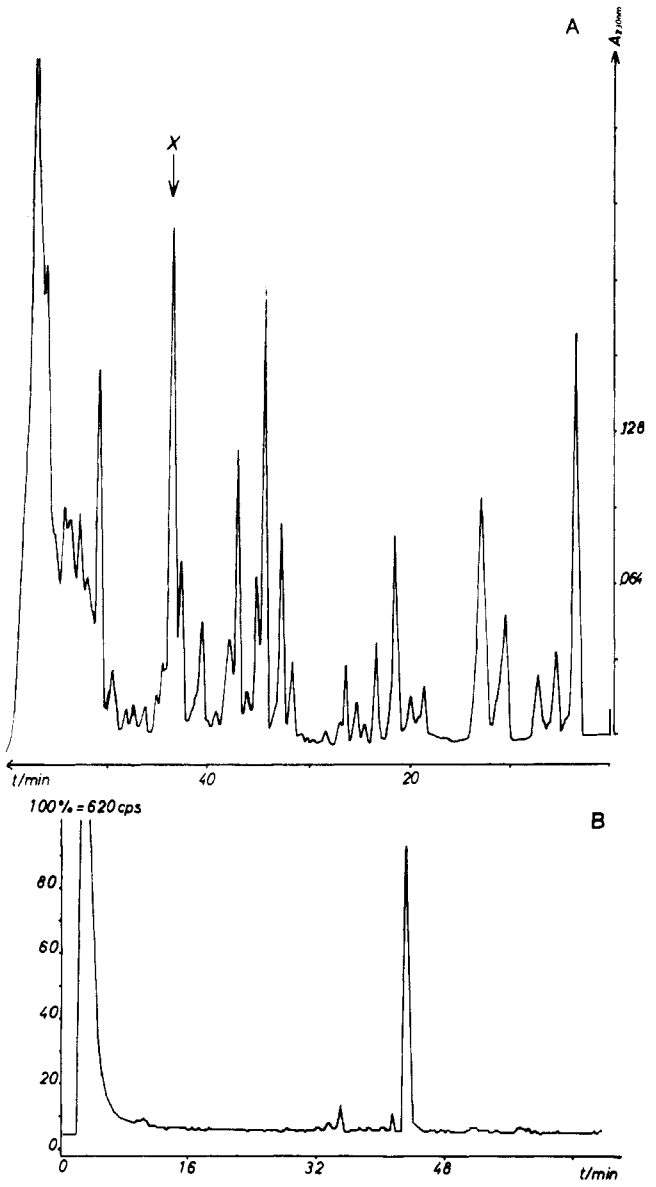

FIGURE 3: Reversed-phase chromatography of Lys-C digest of ^{32}P-enzyme I. (A) UV absorption: wavelength 230 nm, 0.32 absorbance unit full scale, chart speed 200 mm/h, MN Nucleosil C8 column, 40 °C, elution in phosphate buffer system. The X marks the radioactivity containing peptide. Its retention time is 43.5 min. (B) Radioactivity. Here the retention time of the active center peptide was found to be 43 min 4 s. The slight difference is caused by the capillary volume between the detectors.

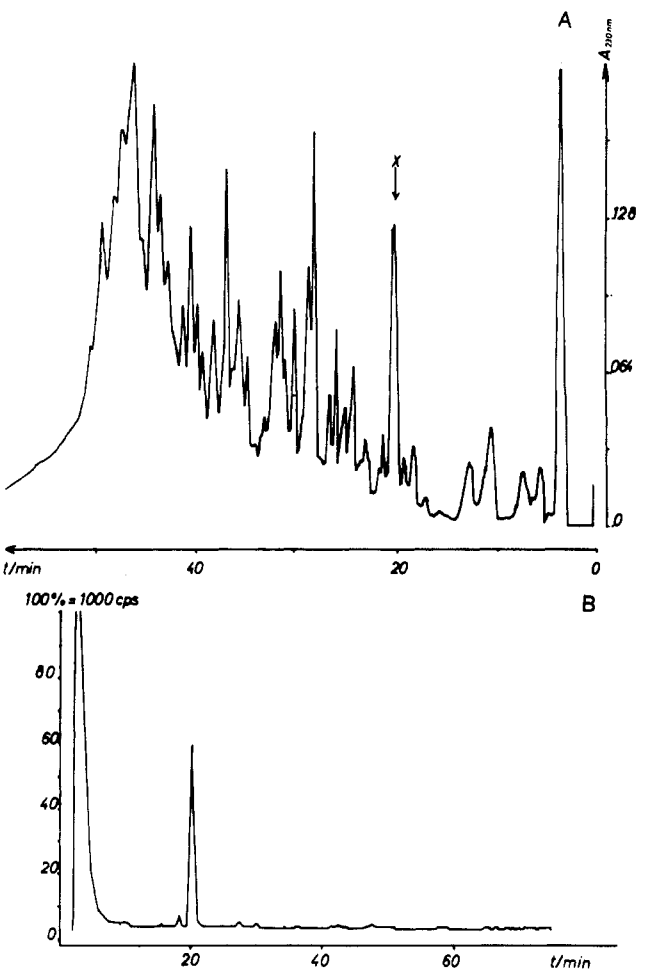

FIGURE 4: Reversed-phase chromatography of a tryptic digest of ^{32}P-enzyme I. (A) UV absorption (conditions as in Figure 3A). The retention time of the active center peptide is 20.4 min. (B) Radioactivity. The retention time is 20.1 min.

reversed-phase HPLC now allows isolation of analytically pure peptides with good yield often in a single separation run (Lottspeich et al., 1981). Previous attempts to obtain the active center failed due to their insensitivity which did not allow the localization of peptides by UV absorption in dilute solution.

For isolation of the active center peptide, 1 mg of enzyme I was incubated with [^{32}P]PEP as described under Materials and Methods. For digestion of ^{32}P-enzyme I, 50 μg of trypsin, Lys-C protease, or Arg-C protease (clostripain) was added. The peptide mixture was separated by HPLC on a reversed-phase column using an acetonitrile gradient in 20 mM potassium phosphate, pH 6.8. Figure 3A shows the peptide map of ^{32}P-enzyme I following digestion with Lys-C protease. One peptide is marked with an X. This was the only major labeled peptide as can be seen from Figure 3B. The radioactive peak with low retention time is due to [^{32}P]PEP and phosphate, which is hydrolyzed from ^{32}P-enzyme I. Figure 4A shows the peptide map and Figure 4B the radioactivity profile after HPLC separation of ^{32}P-enzyme I digested with trypsin. Again only one labeled peptide was found which had a much lower retention time compared to the labeled peptide obtained with Lys-C protease. Cleavage with Arg-C protease gave a labeled peptide which migrated very similar to the labeled peptide obtained with trypsin (retention time 21 min, data not shown). The labeled peptides were collected and rechromatographed on the same column using an acetonitrile gradient in the presence of 0.1% TFA, pH 2.0.

The 3-phosphohistidine seemed to to be much more acid stable in the peptide compared to the active enzyme. After the second chromatography, we therefore observed one major radioactive peak besides the minor phosphate peak. According to UV absorption, the labeled peptide was homogeneous. The peptide obtained with Lys-C protease had a retention time of 43 min, whereas the peptide obtained with trypsin or Arg-C protease had a retention time of 21 min (data not shown). The solvent of the second HPLC run was evaporated, and the amino acid composition of the different peptides was determined. Table II summarizes the results obtained for two different experiments with trypsin or clostripain and for one experiment with Lys-C protease. The results indicate that the peptides obtained with trypsin or Arg-C protease are identical whereas the peptide isolated from the Lys-C digest was larger. In all peptides, only one histidyl residue was found. The amino acid sequence of the active center peptide obtained with Lys-C protease was next determined. It consists of the following 29 amino acids: -Ala-Phe-Val-Thr-Asp-Ile-Gly-Gly-Arg-Thr-

Table II: Amino Acid Composition of Active Center Peptides of Enzyme I from *Streptococcus faecalis*[a]

	trypsin	trypsin	clostripain	clostripain	Lys-C	Lys-C/T1	Lys-C/T2	Lys-C/T3[b]
Asp	0.2	0.3			1.2	1.0		
Thr	0.7	1.0	0.1	1.0	2.9	1.2	1.0	1.0
Ser	0.8	1.5	0.8	1.3	3.1		2.1	1.2
Glu					0.9			1.2
Pro					0.7			1.0
Gly	0.9	0.3	0.4	0.3	3.2	1.9		1.3
Ala	1.6	1.7	1.7	1.6	4.0	1.0	2.0	1.1
Val	0.2	0.3	0.1	0.1	1.8	0.9		0.8
Met	0.6	0.5	0.6	0.7	0.7		0.7	
Ile	0.8	0.8	0.7	0.8	3.7	1.0	1.2	1.8
Leu	0.5				0.8			1.1
Phe					1.2	0.9		
Lys					0.9			1.0
His	1.0	1.0	1.0	1.0	1.3		0.9	
Arg	1.1	0.9	1.1	1.2	2.2	1.1	1.3	

[a] Compositions obtained with three different proteases: Lys-C, trypsin, and clostripain. For trypsin and clostripain, the results of two different experiments are shown. Numbers are expressed as moles of amino acid per mole of peptide. [b] Lys-C peptide cleaved with trypsin.

Ser-His*-Ser-Ala-Ile-Met-Ala-Arg-Ser-Leu-Glu-Ile-Pro-Ala-Ile-Val-Gly-Thr-Lys-. From the sequence, it can be seen that the Arg-C protease clostripain and trypsin must produce the same active center peptide (positions 10–18 of the Lys-C peptide). The sequence showed only one histidyl residue in position 12. The Lys-C active center peptide was further cleaved with trypsin, and the digest was separated by HPLC on a reversed-phase column. Three peptides were obtained. Amino acid analysis of these peptides is summarized in Table II.

Lys-C/T2 was found to be identical with the active center peptide obtained with trypsin or Arg-C protease. The other two peptides did not contain a histidyl residue, further indicating that the Lys-C active center peptide contains only one histidyl residue. We therefore conclude that histidine in position 12 of the Lys-C active center peptide carries the phosphoryl group in P-enzyme I. The peptide does not fit into the first 59 amino acids of the N-terminal part of enzyme I.

Discussion

So far, there is only one well-characterized phosphoenolpyruvate-dependent protein kinase, which is enzyme I of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Enzyme I is phosphorylated at the N-3 position of a single histidyl residue.

Phosphohistidine was found as an intermediate in many phosphoryl transfer reactions (Schneider, 1978) and also as an intermediate in a protein kinase reaction. The ATP-dependent histone kinase (pig brain) was found to contain a phosphohistidine (Kochetkov et al., 1976). In contrast to the bacterial cells, there is only one report of a PEP-dependent protein kinase in eukaryotic cells. The soluble fraction of rat skeletal muscle was shown to contain a PEP-dependent protein kinase, which phosphorylated one major protein of molecular weight 25 000 according to $NaDodSO_4$ gels (Khandelwal et al., 1983). The 25 000-dalton protein was phosphorylated at a seryl residue. The PEP-dependent kinase was found to be activated by cytidine 5′-triphosphate (Mattoo et al., 1984).

Enzyme I of the bacterial PTS phosphorylates HPr, and as was shown for *E. coli*, the fructose induced HPr like protein FPr (Waygood et atl., 1979). The phosphorylation site was found to be His-15 in HPr of *Staphylococcus aureus* (Beyreuther et al., 1977), *S. typhimurium* (Weigel et al., 1982), and *S. faecalis* and *Bacillus subtilis* (Muss, 1982). Detailed studies on substrates of ATP-dependent protein kinases in eukaryotic cells revealed some common features of the phosphorylation site (Krebs & Beavo, 1979). Thus, lysyl or arginyl residues located close to the phosphorylation site seem to play an important role. For Ca^{2+}-stimulated protein kinases, the sequence -Lys-Glu-Ile-Ser(P)-Val-Arg- was found to be essential (Tessmer et al., 1977). In particular, the arginyl residue in the second position C-terminal to the phosphorylatable serine was of critical importance. In HPr of *S. aureus*, *S. faecalis*, and *B. subtilis*, the amino acid sequence around His-15 was -Gly-Ile-His-Ala-Arg-Pro-Ala-Thr- (Muss, 1982), whereas in HPr of *S. typhimurium* and *E. coli* it was Gly-Leu-His-Thr-Arg-Pro-Ala-Ala (Weigel et al., 1982; Stüber, 1983). Modification of the arginyl residue with 1,2-cyclohexanedione in HPr of *S. faecalis* and of *E. coli* almost completely abolished PEP-dependent phosphorylation by enzyme I (Muss, 1982; Dörschug, 1982). The arginyl residue, two amino acid residues behind the phosphorylation site, seemed to be essential for PEP-dependent phosphorylation of HPr as was the arginyl residue in the substrates of some Ca^{2+}-stimulated ATP-dependent protein kinases. P-HPr transfers the phosphoryl group to the N-3 position of a histidyl residue of a factor III protein (Kalbitzer et al., 1981; Dörschug et al., 1984).

As enzyme I and factor III proteins interact with P-HPr and as both proteins carry the phosphoryl group bound to the N-3 position of a histidyl residue, similarities of their active sites could be expected. The active center of factor III^{Glc} of the *E. coli* PTS was found to be -Val-His-Phe-Gly-Ile-Asp- (Dörschug et al., 1984), and the active center of factor III^{Lac} of *S. aureus* PTS was -Gly-Gln-Asp-His-Leu-Met-Thr-Thr- (Deutscher et al., 1982).

The active sites of a factor III protein and of enzyme I from two closely related organisms show no similarities in their primary structure (Stüber et al., 1985). It is interesting to note that HPr of the PTS of Gram-positive bacteria is phosphorylated not only by enzyme I in a PEP-dependent reaction at a histidyl residue but also by an ATP-dependent protein kinase at a seryl residue (Deutscher & Saier, 1983). The ATP-dependent protein kinase has recently been purified from *S. faecalis* (Deutscher & Engelmann, 1984).

The product of the ATP-dependent protein kinase catalyzed reaction, P-Ser-HPr, was found to be a poor substrate of enzyme I. However, in the presence of factor III proteins, P-Ser-HPr was found to be phosphorylated by PEP and enzyme I much faster than in the absence of factor III proteins (Deutscher et al., 1984). These results indicate that both factor III proteins and enzyme I must interact with P-Ser-HPr in a different way which requires two distinct active sites of enzyme I and of factor III. Our sequence data of the active sites of enzyme I and factor III are in agreement with this suggestion. To further support these findings, we are isolating

the active-site peptide of factor III specific for gluconate from the same *S. faecalis* strain from which enzyme I was prepared.

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