THE STAPHYLOCOCCAL PEP DEPENDENT PHOSPHOTRANSFERASE SYSTEM: DEMONSTRATION OF A PHOSPHORYLATED INTERMEDIATE OF THE ENZYME I COMPONENT

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Received 8 March 1974

1. Introduction

The PEP dependent phosphotransferase system (PTS) is responsible for the vectorial phosphorylation of various carbohydrates in bacteria [1].

The phosphorylation of galactosides in *Staphylococcus aureus* occurs as follows [2–4]:

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

$$\text{HPr-P+1/3 F III} \rightleftharpoons \text{1/3 F III-P+HPr} \quad (2)$$

$$\text{1/3 F III-P+galactoside} \xrightarrow[\text{Mg}^{2+}]{\text{Enzyme II}} \text{galactoside-6-P+1/3 F III} \quad (3)$$

Roseman and coworkers postulated a phosphorylated Enzyme I (E I), intermediate during the phosphorylation of HPr. However experiments which proved the existence of such an intermediate have not yet been presented. In this paper we describe the isolation and partial characterisation of the phosphorylated E I intermediate.

2. Materials and methods

2.1. *^{32}P Labelled phosphoenolpyruvate (^{32}PEP)*

A chemical synthesis of ^{32}PEP starting from β-chlorolactic acid and ^{32}P orthophosphate (Buchler, Braunschweig) has been described previously [5]. The specific activity of the ^{32}PEP was 1–5 mCi/μM.

2.2. *Protein components of the PTS*

2.2.1. Enzyme I

We used a partially purified preparation. As in various other laboratories, we were not able to obtain homogeneous E I from *S. aureus*.

The following purification procedures were used. A crude extract prepared from cells washed with EDTA (60 g of the strain S 305 A) [6] was chromatographed on DEAE-Sephadex A25. E I activity was eluted by a salt gradient 0–0.5 M NaCl in standard buffer (Tris–HCl, 0.05 M, pH 7.5, 0.1% mercaptoethanol). Fractions containing enzymatic activity were applied to a hydroxyapatite column prepared according to Atkinson et al. [7]. E I eluted from 0–0.05 M potassium phosphate in standard buffer. E I-containing fractions were concentrated by pressure dialysis (Amicon), and further purified on a column of Sephadex G 150 eluted with standard buffer. The recovery of E I activity was around 20%, the purification about 25-fold. The preparation still showed several protein bands when subjected to acrylamide gel electrophoresis.

2.2.2. Factor III (F III) specific for galactosides

A detailed purification procedure yielding homogeneous protein has been described [6].

2.2.3. HPr

HPr was a homogeneous preparation obtained after the following steps. The supernate from the acid precipitation of the F III purification [6] was fractionated further by DEAE-cellulose chromatography and molecular sieving. Details of the purification procedure will be published elsewhere.

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2.2.4. Enzyme II specific for galactosides

The enzyme used for the phosphorylation of the galactoside was a washed membrane preparation as described earlier [8].

Prior to use all protein preparations were assayed to contain the desired biological activity using the assay system based on the complementation of PTS mutant extracts [9].

2.3. *Paper electrophoresis*

The separation of sugar phosphates, PEP and P_i was achieved on a pherograph according to Wieland and Pfleiderer (Hormuth and Vetter, Heidelberg, Germany), using 0.05 M triethylammonium acetate, pH 4.5, and Whatman 3 paper at 60 V/cm.

^{32}P-containing spots were localized by autoradiography with X-ray film.

3. Results and discussion

3.1. *Isolation of the phosphorylated Enzyme I intermediate*

A purified E I preparation was incubated with ^{32}PEP. The mixture was separated on a Sephadex G 75 column, radioactivity appeared in the void volume of the column, superimposed by the E I activity, the phosphorylation of the protein required Mg^{2+} ions as shown in fig. 1.

The isolated E I-P intermediate was subjected to high voltage paper electrophoresis at pH 4.5. The radioactivity did not leave the starting line as expected for a ^{32}P-labelled macromolecule. Traces of PEP or P_i could not be detected on the autoradiograph (compare fig. 3).

3.2. *Specific phosphoryl-transfer from the Enzyme I–P intermediate to the HPr component and to a galactoside*

Since we were not able to obtain pure E I from *S. aureus*, the specificity of the phosphorylated intermediate had to be proven. Two specific features were chosen.

1) The isolated E I–P intermediate was used to phosphorylate the protein HPr, its only known substrate as indicated in eq. 1 of the reaction scheme. The experiments described in fig. 2 show nearly quantitative phosphate transfer from E I–P to HPr.

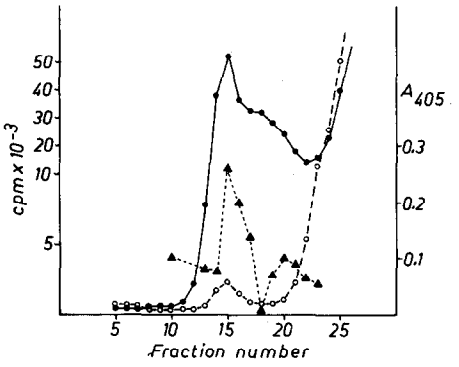

Fig. 1. Five mg of enzyme I in 0.05 M Tris–HCl buffer, pH 7.5, containing 0.1% mercaptoethanol was incubated with 0.5 ml of ^{32}PEP solution (about 10^8 cpm) at 37°C for 10 min. The mixture was separated on a Sephadex G 75 column, 1.6 X 33 cm, with the solution buffer described above. Fractions of 2 ml were collected. 0.05 ml aliquots were counted with a Packard scintillation counter using scintigel (Roth, Karlsruhe, Germany) as counting fluid. 0.05 ml aliquots were tested for E I activity. (●——●) The incubation mixture contained 10^{-2} M $MgCl_2$; (○——○) the incubation mixture contained 10^{-2} M EDTA; (▲——▲) E I activity tested with the corresponding mutant extract.

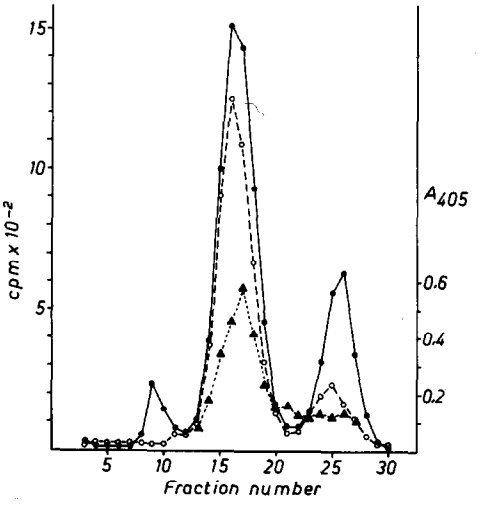

Fig. 2. 0.8 ml of the fractions 14–16 (compare fig. 1) were incubated with 400 μg of HPr for 10 min at 37°C. The mixture was separated on a Sephadex G 75 column, 1.5 X 28 cm, with 0.1 M $NaHCO_3$ pH 8.8, as elution buffer. Fractions of 2 ml were collected. Samples of 0.1 ml were counted to determine radioactivity; 0.05 ml aliquots were assayed with the corresponding mutant extract to obtain the activity profile for HPr. (○——○) The incubation mixture contained 10^{-2} M $MgCl_2$; (●——●) the incubation mixture was treated with 10^{-2} M EDTA; (▲——▲) HPr activity assayed with the mutant extract.

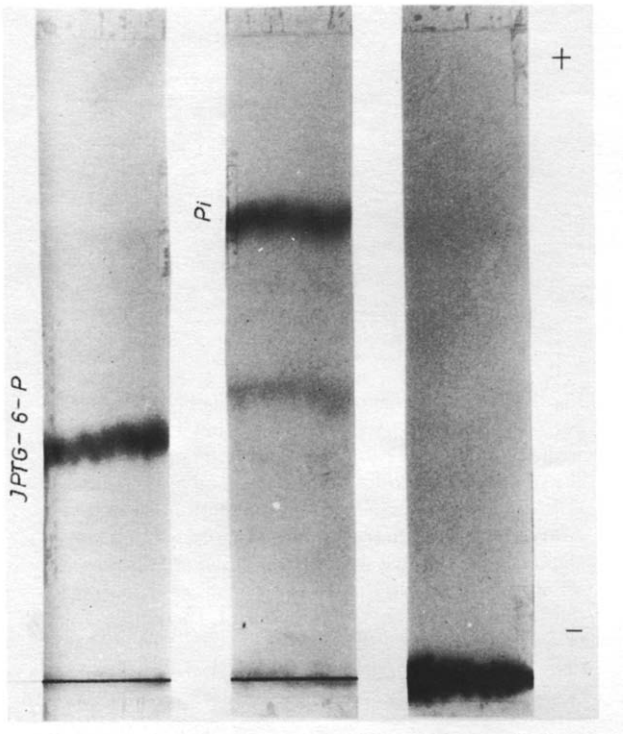

Fig. 3. 0.1 ml of the fractions containing ^{32}P-labelled phosphoryl enzyme I were incubated with 40 μg HPr, 50 μg Factor III and 10 μmoles of $MgCl_2$ at 37°C for 15 min. The total volume of the mixture was 0.2 ml. Aliquots of 0.05 ml were subjected to high voltage paper electrophoresis at pH 4.5; radioactive spots were localised by autoradiography. Left sample: autoradiograph from experiment containing 2 μmoles of IPTG: formation of IPTG-6-phosphate. Middle sample: same experiment as above without IPTG: formation of inorganic phosphate. Right sample: undiluted phosphoryl enzyme I used in the described experiments without the addition of HPr, F III and E II.

The peak at fraction 25 indicating P_i may be due to hydrolysis of HPr-P during the reaction time. Fig. 2 further demonstrates that the phosphoryl transfer to HPr did not require Mg^{2+} ions. The reaction occurs as well in 10^{-2} M EDTA. The amount of HPr used in our experiment was big enough to assay the biological activity of HPr, as shown in fig. 2.

2) The isolated E I–P intermediate produced galactoside-6-phosphate if incubated with purified HPr, F III and E II as shown in fig. 3. The ^{32}P phosphoryl group is transferred to the thiogalactoside IPTG (isopropyl-β-thiogalactoside) which is a good substrate of the staphylococcal PTS.

3.3. *Stability of the phosphoryl enzyme I intermediate*

In comparison to HPr–P where the phosphoryl group is bound to the N_1 of the histidine residue (half life at pH 4, 37°C, 4 min), phosphoryl enzyme I is much more stable at low pH (half life at pH 2.5, 37°C, 100 min). No detectable loss of phosphate has been found after 30 min at pH 12.2, 37°C.

Conclusions about the nature of the phosphoryl linkage in the enzyme I protein cannot be drawn at the present stage of this research. However our experimental results allow us to give a more detailed description of the reaction described in eq. 1 as follows:

$$\text{PEP+E I} \xrightarrow{Mg^{2+}} \text{E I-P+pyruvate}$$

$$\text{E I-P+HPr} \rightleftharpoons \text{HPr-P+E I.}$$

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