Preparation and Properties of a Phosphoenzyme from the Phosphoglucumutase of *Micrococcus lysodeikticus*

By JOHN B. CLARKE, MICHAEL BIRCH and HUBERT G. BRITTON
Department of Physiology, St. Mary's Hospital Medical School, London W2 1PG, U.K.

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1. Phosphoglucumutase from *Micrococcus lysodeikticus* was incubated with ^{14}C - and ^{32}P -labelled glucose 1,6-diphosphate and separated from the cofactor on a Sephadex column. ^{32}P -labelled phosphate (0.7 mol/mol of enzyme) was associated with the enzyme, but no ^{14}C label was. 2. The ^{32}P -labelled enzyme exchanged its label with the substrates. When the labelled enzyme was incubated in Tris buffer, pH 8.3, at 30°C the proportion of exchangeable label slowly fell indicating a half-life of the phosphoenzyme of about 50 h. 3. When HClO_4 was added to the labelled phosphoenzyme all of the label was precipitated with the protein and none was released as P_i . On alkaline hydrolysis P_i was released at a rate comparable with the rate of hydrolysis of the phosphoenzyme from rabbit muscle. 4. We conclude that the phosphoenzyme from *Micrococcus lysodeikticus* yields a relatively stable, catalytically active phosphoenzyme when treated with cofactor, and that there is no evidence for the formation of an enzyme-glucose 1,6-diphosphate complex. The properties of the phosphoenzyme, which resemble those of rabbit muscle phosphoglucumutase, suggest that the phosphate may be bound to serine.

Phosphoglucumutases have been isolated from a variety of sources and all require glucose 1,6-diphosphate as a co-factor. The enzyme from rabbit muscle has been intensively investigated. It was isolated as a phosphoenzyme by Jagannathan & Luck (1949) and Ray & Roscelli (1964*a,b*) obtained parallel lines when they plotted the reciprocals of the initial velocities against the reciprocal of the glucose 1-phosphate concentration at different glucose 1,6-diphosphate concentrations and vice versa. The latter workers interpreted their kinetic findings as evidence for a phosphoenzyme mechanism in which the substrates react with the phosphoenzyme and the function of the cofactor is to phosphorylate the enzyme. Britton & Clarke (1968) with isotopic measurements obtained further strong evidence for a phosphoenzyme mechanism, although they pointed out that none of the kinetic evidence entirely excludes the possibility of an enzyme-glucose 1,6-diphosphate complex in which one of the phosphates of the complex behaves as the phosphate of a phosphoenzyme. In this context Hashimoto & Handler (1966) have claimed that an enzyme-glucose 1,6-diphosphate complex may be obtained if mild conditions are used.

Handler and his co-workers investigated phosphoglucumutase from a number of sources. The flounder and shark muscle mutase (Hashimoto & Handler, 1966) and the mutase from *Escherichia coli* (Joshi & Handler, 1964) could be isolated as phosphoenzymes and they gave parallel-line reciprocal initial-velocity plots. However, the enzyme from

Micrococcus lysodeikticus and *Bacillus cereus* (Hanabusa *et al.*, 1966) could not be phosphorylated. No glucose monophosphate was formed when glucose 1,6-diphosphate was added to substrate quantities of the enzyme; and no labelled phosphoenzyme could be detected after incubation of the enzyme with glucose 1,6- ^{32}P diphosphate and separation of the enzyme by acid or $(\text{NH}_4)_2\text{SO}_4$ precipitation or by dialysis. Similarly, Alpers (1969) was unable to phosphorylate the enzyme from *M. lysodeikticus* with 1,3-diphosphoglycerate although the rabbit muscle enzyme is phosphorylated by this agent. Further, with both enzymes Hanabusa *et al.* (1966) obtained reciprocal initial-velocity plots consisting of families of converging lines, which they interpreted as being kinetic evidence that both the substrate and glucose 1,6-diphosphate had to be present simultaneously on the enzyme molecule. They therefore proposed that these bacterial enzymes do not possess a phosphoenzyme mechanism but catalyse an intermolecular transfer of phosphate from glucose 1,6-diphosphate, i.e. a sequential mechanism. In this context the pH optimum of these enzymes (pH 8.5) differs considerably from that of the muscle mutases (pH 7.4) although the *E. coli* (pH optimum 8.5) which seems to possess a phosphoenzyme mechanism appears to be an exception. This latter mutase also differs from the others in possessing an essential thiol group.

We have pointed out that converging initial-velocity plots do not necessarily exclude a phosphoenzyme mechanism and we have reinvestigated the

mechanisms of the enzyme from *M. lysodeikticus* by isotopic methods (Britton & Clarke, 1972; Clarke & Britton, 1974). Our findings indicate that the enzyme does possess a phosphoenzyme mechanism, although, as we have pointed out in connexion with the rabbit muscle enzyme, the kinetic evidence does not completely exclude the possibility that an enzyme-glucose 1,6-diphosphate complex may be involved which is behaving as a phosphoenzyme. In the present paper we have therefore attempted to isolate the active form of the enzyme by gel filtration after treatment of the enzyme with ^{32}P - and ^{14}C -labelled glucose 1,6-diphosphate. Our results strongly indicate that a true phosphoenzyme is formed.

Experimental

Materials

Glucose 1-phosphate and glucose 6-phosphate as the disodium salts, glucose 1,6-diphosphate as the cyclohexylamine salt, phosphoglucumutase from rabbit muscle [2mg/ml, 100 units/mg in 2.5M-(NH_4) $_2\text{SO}_4$] and glucose 6-phosphate dehydrogenase from yeast [1 mg/ml, 140 units/mg in 3.2M-(NH_4) $_2\text{SO}_4$] were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany).

Methods

Preparation of *M. lysodeikticus* phosphoglucumutase. Freeze-dried cells of *M. lysodeikticus* were obtained either from Miles-Seravac (Maidenhead, Berks., U.K.) or from the Microbial Research Establishment (Porton Down, Wilts., U.K.). The phosphoglucumutase was isolated by the method of Hanabusa *et al.* (1966). At each stage of the preparation the activity of the enzyme was only about 4% of that described by these authors. The purified and desalted enzyme was stored in 50mM-Tris-acetate buffer, pH 7.0, containing 5.0mM-MgCl $_2$ at 4°C.

Preparation of glucose 1,6-[^{32}P]diphosphate. ^{32}P label was incorporated into glucose 6-phosphate and glucose 1-phosphate by the technique described by Britton & Clarke (1968) for the preparation of glucose 1-[^{32}P]phosphate, with the additions of 100 μl of rabbit muscle phosphoglucumutase (2mg/ml), 5nmol of glucose 1,6-diphosphate and 0.2ml of histidine-Tris-Mg $^{2+}$ buffer, pH 7.4 (histidine, 40mM, Tris-HCl, 12.0mM, MgCl $_2$, 1.1mM), to the reaction mixture. Glucose 1-[^{32}P]phosphate and glucose 6-[^{32}P]phosphate were separated by paper chromatography from [^{32}P]P $_i$ and then approx. 0.2 μmol of the radioactive monophosphates was incubated with 1 μmol of glucose 1,6-diphosphate, 0.2ml of histidine-Tris-Mg $^{2+}$ buffer, pH 7.4, and 0.01 ml of rabbit muscle phosphoglucumutase (2mg/ml) in a final volume of 0.4ml for 1h at 30°C. Glucose 1,6-[^{32}P]diphosphate was separated by chromato-

graphy on DEAE-cellulose paper (Whatman DE 81) for 6h with 0.12M-NH $_3$. After elution with 5M-NH $_3$ it was freeze-dried and stored at -20°C. It had a specific radioactivity of about 25 $\mu\text{Ci}/\mu\text{mol}$.

Preparation of [^{14}C]glucose 1,6-diphosphate. High specific radioactivity [^{14}C]glucose 6-phosphate (about 20 μCi , 100-150 $\mu\text{Ci}/\mu\text{mol}$) (The Radiochemical Centre, Amersham, Bucks., U.K.) was incubated with 0.1 μmol of glucose 1,6-diphosphate, 0.01 ml of rabbit muscle phosphoglucumutase (2mg/ml), in 0.25ml of histidine-Tris-Mg $^{2+}$ buffer, pH 7.4, for 2½h at 30°C. [^{14}C]Glucose 1,6-diphosphate was extracted as described for glucose 1,6-[^{32}P]diphosphate and stored at -20°C. It had a specific radioactivity of about 67 $\mu\text{Ci}/\mu\text{mol}$.

Preparation of labelled enzyme. The enzyme (about 10 units in 0.3ml, specific activity 50-100 units/mg) was incubated with [^{14}C]glucose 1,6-diphosphate, (0.28 μCi) and glucose 1,6-[^{32}P]diphosphate (0.19 μCi) in 0.1ml of freshly prepared Tris buffer, pH 8.3 (Tris-acetate, 100mM, cysteine, 25mM, MgCl $_2$, 10mM) for 1 min at 30°C. The solution was immediately cooled to 0°C and applied to a Sephadex column (G-25, 6cm $\times$ 0.9cm) that had been equilibrated with the buffer at 4°C. With the same Tris buffer as eluent, 0.3ml samples were collected, and ^{14}C - and ^{32}P -radioactivity and enzyme activity were assayed. ^{14}C and ^{32}P were determined by differential scintillation counting as described by Britton & Clarke (1968) and enzyme activity was measured as described by Hanabusa *et al.* (1966).

Exchange studies with ^{32}P -labelled enzyme. Labelled enzyme was prepared as described above but no [^{14}C]glucose 1,6-diphosphate was added to the incubation mixture. A fraction (1.0ml) which included the enzyme peak was taken from the Sephadex column and immediately warmed to 30°C. Samples (0.1ml) were taken at various intervals to determine the proportion of [^{32}P]P which was exchangeable with the substrates. To each sample 0.02ml of 50mM-glucose 6-phosphate and 0.01 ml of 100mM-potassium phosphate, pH 7.0, were added and exchange was allowed to take place for 1 min at room temperature. The solution was then cooled to 0°C and 1.70ml of 0.6M-HClO $_4$ was added followed by 1.0ml of freshly prepared phosphate-precipitating reagent [57mM-ammonium molybdate and 74mM-triethylamine, 1:1 (v/v); Sugino & Miyoshi, 1964]. After standing overnight at 4°C the solutions were centrifuged and the radioactivity in the glucose 6-phosphate was determined in 1.0ml of supernatant as described by Britton & Clarke (1968). The total ^{32}P radioactivity was determined by placing 0.1 ml of the original solution in a scintillation vial and counting as described above.

Gel electrophoresis in the presence of sodium dodecyl sulphate. Electrophoresis of the labelled phosphoenzyme was carried out on polyacrylamide

gels containing 0.75% sodium dodecyl sulphate, by the procedure of Marshall & Zamecnik (1969). The gels were sliced into 2mm sections which were placed with 15ml of Bray's (1960) solution in scintillation vials and counted for ^{32}P in a Packard scintillation counter (Packard Instrument Co. Ltd., Wembley, Middx., U.K.).

Addition of acid to ^{32}P -labelled enzyme. ^{32}P -labelled phosphoenzyme (about 1 unit) in a volume of 100 μl was added to 0.85ml of 0.6M- HClO_4 , followed by 0.01ml of 0.1M- Na_2HPO_4 , pH 7.0, and 0.1ml of bovine serum albumin (10mg/ml) all at 4°C. The suspension was centrifuged at 2000g for 10min and the supernatant was removed. After washing with the same volume of HClO_4 and Na_2HPO_4 , the precipitate was dissolved in 0.2ml of 1M- NaOH . Samples (0.2ml) of the supernatant and dissolved precipitate, appropriately neutralized with acid or alkali, were placed in scintillation vials, 15ml of Bray's (1960) solution was added and the vials were counted as described for gel electrophoresis.

Alkaline hydrolysis of ^{32}P -labelled enzyme. The eluate from the Sephadex column containing the phosphoenzyme (about 5 units) was immediately warmed to 37°C and 1M- NaOH was added. Samples (0.2ml) were withdrawn at intervals, cooled to 0°C, and 0.2ml of 0.6M- HClO_4 and 10 μl of 100mM- Na_2HPO_4 were added. After centrifugation at 2000g for 10min 0.3ml samples of the supernatants were taken, and the radioactivity was assayed by scintillation counting as described above.

Determination of specific radioactivity of labelled glucose 1,6-diphosphate. The molar concentrations of the solutions of labelled glucose 1,6-diphosphate were determined by adding 0.4ml of 1M- HCl to 0.4ml of each solution (about 0.5mM) and heating to 100°C in a water bath for 20min to hydrolyse the glucose 1,6-diphosphate to glucose 6-phosphate. Each solution was then cooled and neutralized by the addition of 0.4ml of 1M- NaOH . A sample (0.4ml) was added to 0.5ml of triethanolamine buffer, pH 7.6 (triethanolamine- HCl , 100mM, MgCl_2 , 12.5mM) in a cuvette (10mm light-path); 0.2ml of NADP^+ (3.5mg/ml) was added and the volume was made up to 3.0ml. The E_{340} was measured and 0.01ml of glucose 6-phosphate dehydrogenase (1mg/ml) was added. The concentration of glucose 1,6-diphosphate was calculated from the change in E_{340} . The ^{14}C and ^{32}P radioactivities in the original solutions were determined by scintillation counting as described previously to derive the specific radioactivities.

Results

Evidence for phosphoenzyme formation

Phosphoglucumutase from *M. lysodeikticus* was incubated with ^{14}C - and ^{32}P -labelled glucose 1,6-

diphosphate for 1 min at room temperature in Tris-cysteine- Mg^{2+} buffer, pH 8.3 (Tris-acetate, 100mM, cysteine, 25mM, MgCl_2 , 10mM), cooled to 4°C and applied to a Sephadex G-25 column. When the column was eluted with the same buffer the peak of enzyme activity was associated with a peak of ^{32}P radioactivity but there was no corresponding peak of ^{14}C radioactivity (Fig. 1). When the ^{32}P -labelled enzyme was incubated with an excess of substrates for 1 min at room temperature, 93% of the label exchanged with the substrates. The ^{32}P label associated with the enzyme therefore cannot have been present as $[\text{P}^{32}\text{P}]_i$ but must presumably have been in the form of a phosphoenzyme. Further, the exchange of label with the substrates provides strong evidence that the label was associated with phosphoglucumutase activity. This is of significance since the enzyme preparation (specific activity 50–100 units/mg) was relatively impure. When the phosphoenzyme was electrophoresed (see below) the ^{32}P label migrated with a single band of molecular weight about 70000. Adopting this value and a specific activity of the enzyme of 970 units/mg (Hashimoto & Handler, 1966) the amount of labelled phosphate associated with the enzyme was about 0.7 mol/mol of enzyme.

Fig. 1. Preparation of ^{32}P -labelled phosphoglucumutase from *Micrococcus lysodeikticus* by gel filtration

Enzyme solution (0.3ml, 11 units) was added to [^{14}C]-glucose 1,6-diphosphate (0.28 μCi , specific radioactivity 67 mCi/mmol) and glucose 1,6- $[\text{P}^{32}\text{P}]$ diphosphate (0.19 μCi , specific radioactivity 25 mCi/mmol) in 0.1ml of Tris-cysteine- Mg^{2+} buffer, pH 8.3 (Tris-acetate, 100mM, cysteine, 25mM, MgCl_2 , 10mM) and the mixture was incubated for 1 min at 30°C. The solution was immediately cooled to 0°C and applied to a Sephadex G-25 column previously equilibrated with the Tris buffer. The column was eluted with the same buffer and the fractions (0.3ml) were assayed for ^{14}C and ^{32}P radioactivity and enzyme activity. The unshaded peak represents ^{14}C radioactivity and the shaded peaks ^{32}P radioactivity. The dotted line represents enzyme activity.

Fig. 2. Decay of the ^{32}P -labelled phosphoenzyme in Tris-cysteine- Mg^{2+} buffer, pH 8.3, at 30°C

The buffer contained Tris-acetate, 100mM, cysteine, 25mM and MgCl_2 , 10mM. The percentage of ^{32}P label exchangeable with the substrates was plotted against time. About 10 units of enzyme were used. The symbols $\bullet$ and Δ refer to different experiments.

Fig. 3. Hydrolysis of ^{32}P phosphoenzyme by NaOH at 37°C

The logarithm to base 10 of the percentage of ^{32}P label precipitated by HClO_4 is plotted against time. $\circ$, Hydrolysis in 0.25M- NaOH ; $\bullet$, hydrolysis in 0.33M- NaOH . About 5 units of enzyme were used.

A second experiment with ^{14}C - and ^{32}P -labelled substrates gave an essentially similar result with about 0.5mol of phosphate bound/mol of enzyme but again no binding of ^{14}C label. In the subsequent experiments described below the same procedure was followed without the addition of [^{14}C]glucose 1,6-diphosphate.

Stability of the phosphoenzyme

The stability of the phosphoenzyme in Tris-cysteine- Mg^{2+} buffer, pH 8.3, at 30°C was studied by

taking samples at intervals to determine the proportion of exchangeable phosphate. In two experiments (Fig. 2) there was a slow decline over a period of 4h and if a first-order rate process can be assumed the half-life of the phosphoenzyme would appear to be about 50h.

Nature of the phosphate bond in the phosphoenzyme

The stability of the phosphoenzyme to acid was investigated by the addition of HClO_4 and serum albumin at 4°C . After washing the precipitate with a HClO_4 solution containing P_i , virtually all of the ^{32}P label (99.4%) was found in the precipitate. A similar result was obtained in a second experiment. The enzyme-phosphate bond is therefore stable to acid.

To investigate the stability of the phosphoenzyme to alkali, freshly prepared phosphoenzyme was made 0.25 or 0.35M with respect to NaOH and incubated at 37°C for 4h. Samples were taken for the determination of [^{32}P] P_i . The results (Fig. 3) indicated hydrolysis according to a first-order reaction with a half-life of about 2.4h in 0.25M- NaOH and 1.7h in 0.33M- NaOH .

Electrophoresis of the phosphoenzyme

Electrophoresis of the labelled phosphoenzyme was carried out in polyacrylamide gels containing 0.75% sodium dodecyl sulphate by the procedure of Marshall & Zamecnik (1969). Rabbit IgG,* mol.wt. 140000, bovine serum albumin, mol.wt. 68000, hen's-egg-white lysozyme, mol.wt. 45000, and lactate dehydrogenase, mol.wt. 36000, were run as standards. The enzyme preparation proved to be inhomogeneous but the ^{32}P label was found to be associated with a single band with a molecular weight of about 70000. There was no evidence of any labelled subunits.

Discussion

The present experiments in which a phosphoenzyme has been isolated provide strong confirmation of our previous kinetic evidence (Britton & Clarke, 1972; Clarke & Britton, 1974) that the phosphoglucomutase from *M. lysodeikticus* has a phosphoenzyme mechanism, and they provide strong evidence that the active form of the enzyme is a true phosphoenzyme rather than an enzyme-glucose 1,6-diphosphate complex. The possibility that an enzyme-glucose 1,6-diphosphate complex may be formed, but that it dissociates during its passage through the Sephadex column into glucose

* Abbreviation: IgG, immunoglobulin G.

monophosphate and phosphoenzyme is not completely excluded, but the complete absence of any ^{14}C label associated with the phosphoenzyme makes this seem unlikely. Further, because the phosphoenzyme exchanges with the substrates it would be necessary to assume that the dissociation is reversible or, alternatively, that the phosphoenzyme is also an active species in the mutase reaction.

The phosphoenzyme is remarkably stable with a half-life of about 50h at 30°C and it is surprising therefore that it has not been previously isolated. The stability of the enzyme-phosphate bond to acid would appear to exclude histidyl, aspartyl or glutamyl phosphates but would be compatible with a seryl phosphate. In rabbit muscle phosphoglucomutase the phosphate is known to be attached to serine (Anderson & Jolles, 1957; Kennedy & Koshland, 1957). Kennedy & Koshland (1957) found a half-life of 1.5h for the hydrolysis of the rabbit muscle phosphoenzyme in 0.5M-NaOH at 37°C. In the present work with the phosphoenzyme from *M. lysodeikticus* values of 2.4h and 1.7h in 0.25M- and 0.33M-NaOH respectively were obtained at 37°C and extrapolation would indicate a value of about 1.2h in 0.5M-NaOH. The similarity of this value to that found for the rabbit muscle enzyme suggests that not only is phosphate bound to serine in the bacterial enzyme but that the environment of serine may be similar to that in the rabbit muscle enzyme. Further evidence for the involvement of a serine and for the similarity of the amino acid sequence around the serine to that of the rabbit

muscle enzyme is presented in a related paper (Milstein *et al.*, 1973).

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