The Mechanism of Phosphoglucumutase from *Micrococcus lysodeikticus*

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(Received 29 June 1973)

The mechanism of the phosphoglucumutase from *Micrococcus lysodeikticus* was investigated. Induced-transport tests at low substrate concentrations (0.15 mM) showed co-transport of the ^{32}P label but no induced transport of the ^{14}C label, which is in quantitative agreement with a phosphoenzyme mechanism with a rapid isomerization of the phosphoenzyme. The results excluded an intramolecular transfer of phosphate and could only have been compatible with a sequential mechanism if the K_m for glucose 1-phosphate had been over 20 times smaller than the measured value. The results of induced-transport tests at intermediate concentrations (1 mM) with both labels agreed quantitatively with a phosphoenzyme mechanism, and induced-transport tests with ^{14}C -labelled substrates at high concentrations (26 mM) indicated that the rate constants for isomerization of the phosphoenzyme must be greater than about $3 \times 10^6 \text{ s}^{-1}$. Consistent with these findings is the fact that ^{14}C label exchanged between the substrates twice as rapidly as the ^{32}P label at chemical equilibrium. Further, since the ^{14}C label exchanged between the substrates about ten times more rapidly than between the substrates and glucose 1,6-diphosphate, glucose 1,6-diphosphate is not an obligatory intermediate in the interconversion of the substrates. It is concluded that, contrary to previous evidence, the mechanism of the enzyme from *M. lysodeikticus* is essentially that of the rabbit muscle enzyme. To account for the rapid isomerization of the phosphoenzyme in both cases a mechanism is proposed in which there is no formal isomerization of the phosphoenzyme.

Rabbit muscle phosphoglucumutase has been intensively investigated and there is strong kinetic evidence to indicate that it possesses a phosphoenzyme mechanism (Scheme 1a) (Ray & Roscelli, 1964a,b; Britton & Clarke, 1968). The mechanism as conventionally illustrated in Scheme 1 involves the participation of a true phosphoenzyme. However,

the kinetic evidence does not completely exclude a variant of this mechanism in which an enzyme–glucose 1,6-diphosphate complex is formed and one of the phosphates of the glucose 1,6-diphosphate performs the role of the phosphate in the phosphoenzyme. Although the rabbit muscle enzyme may be isolated as a phosphoenzyme (Jagannathan & Luck,

Scheme 1. Possible mechanisms for the phosphoglucumutase reaction

(a) illustrates the phosphoenzyme mechanism. The enzyme E has two bonds representing alternative positions for phosphate attachment. The dashed arrows represent slow reactions. (b) is the sequential addition mechanism with the enzyme catalysing an intermolecular transfer of phosphate from glucose 1,6-diphosphate. (c) represents the intramolecular transfer of phosphate. In this case glucose 1,6-diphosphate must activate indirectly.

1949), Hashimoto & Handler (1966) claimed that an enzyme-glucose 1,6-diphosphate complex may be obtained if milder conditions are used.

The phosphoglucomutases isolated by Hanabusa *et al.* (1966) from *Micrococcus lysodeikticus* and *Bacillus cereus*, like the rabbit muscle enzyme, require glucose 1,6-diphosphate for activity and catalyse the exchange of ^{32}P between glucose 1,6-diphosphate and the substrates. Hanabusa *et al.* (1966), however, obtained converging rather than parallel lines with these enzymes when they plotted the reciprocals of the initial velocities against the reciprocals of glucose 1-phosphate concentration at constant glucose 1,6-diphosphate concentration and vice versa. They interpreted these findings as evidence that both the substrate and cofactor had to be present simultaneously on the enzyme molecule. They therefore proposed that the mechanism involves the intermolecular transfer of phosphate from glucose 1,6-diphosphate to the substrates (Scheme 1a) as suggested for the phosphoglucomutase reaction by Cleland (1963). In keeping with this mechanism Hanabusa *et al.* (1966) were unable to detect any glucose monophosphate formation when glucose 1,6-diphosphate was added to substrate quantities of these enzymes, as would be expected if phosphoenzyme formation occurred; nor could they detect any phosphoenzyme when the enzyme was incubated with glucose 1,6- ^{32}P -diphosphate and separated by acid or $(\text{NH}_4)_2\text{SO}_4$ precipitation, or by dialysis. Similarly, Alpers (1969) was unable to phosphorylate the enzyme with 1,3- ^{32}P -diphosphoglycerate although the rabbit muscle is phosphorylated by this agent. These differences indicated that there may be two groups of phosphoglucomutases, those typified by the rabbit muscle enzyme and those typified by the

activity in the substrate is followed during the ensuing chemical reaction. If the flows of radioactive and non-radioactive material are independent of each other the total radioactivity in the substrate will not change during the chemical reaction. However, if there should be an interaction between the flows, the radioactivity will either be transiently carried in the direction of the chemical reaction (co-transport) or in the opposite direction (counter-transport). Comparatively few molecular mechanisms will produce such interaction and the interpretation of the results is therefore relatively unambiguous (Britton, 1965, 1966, 1967; Britton & Clarke, 1968).

To transfer a ^{32}P -labelled phosphate group from glucose 1-phosphate to glucose 6-phosphate by Scheme 1(a) requires that two molecules of glucose 1-phosphate should react in succession with the phosphoenzyme, because the first molecule transfers the label only to the phosphoenzyme. There is thus an intermediate site, namely the phosphoenzyme, in the transfer of phosphate by Scheme 1(a). This indirect transfer will give rise to co-transport of phosphate. In contrast the glucose moiety of the glucose 1-phosphate molecule is transferred directly to the product. With Scheme 1(b) there are two intermediate sites in the transfer of phosphate and one intermediate site in the transfer of the glucose moiety of the glucose 1-phosphate, and co-transport of both phosphate and glucose moieties should occur. With Scheme 1(c) both phosphate and glucose moieties are transferred directly and co-transport should not occur with either label. Note that in this context the term 'intermediate site' does not refer to transient intermediate forms of the enzyme-substrate complex. The overall enzyme reaction may be formally represented by the steps:

bacterial enzymes from *M. lysodeikticus* and *B. cereus*.

The flux approach (Britton, 1965, 1966, 1967; Britton & Clarke, 1968) leads to tests for the mechanisms of the mutases that are independent of initial-velocity measurements. These consist of induced-transport tests with ^{14}C - and ^{32}P -labelled substrates and the comparison of the rates of exchange of ^{14}C - and ^{32}P -labelled substrates at chemical equilibrium. In the induced-transport test the appropriately labelled substrate is allowed to come into equilibrium with its product in the presence of the enzyme, and then unlabelled substrate is added. The total radio-

In Scheme 1(a), E_1 and E_2 represent different forms of the phosphoenzyme or enzyme-glucose 1,6-diphosphate complex. In Scheme 1(b), E_1 and E_2 represent different forms of an enzyme-glucose 1,6-diphosphate complex (see Britton & Clarke, 1972b). This is because ^{32}P -labelled phosphate exchanges much more rapidly between glucose 1-phosphate and glucose 6-phosphate than between glucose 1,6-diphosphate and the monophosphates (rabbit muscle; Ray & Roscelli, 1964a; Hashimoto & Handler, 1966; *M. lysodeikticus*; the present paper). When the isomerization $\text{E}_1 \rightleftharpoons \text{E}_2$ is rapid the degree of co-

transport shown by the different schemes should be independent of all rate constants and dependent only on the concentration of substrates and the equilibrium constant. When the step $E_2 \rightleftharpoons E_1$ is slow the rate constants influence the theoretical patterns through a single parameter α . α is defined by the equation:

$$\alpha = k_{+1}k_{+2}/k_{-3}(k_{-1} + k_{+2}) \quad (3)$$

Further discussion of these tests is given elsewhere (Britton & Clarke, 1968; Britton & Clarke, 1972*b*; Britton *et al.*, 1971, 1972).

A brief account of the present work has been published (Britton & Clarke, 1972*a*).

Experimental

Except where indicated below, reagents and methods were as previously described (Britton & Clarke, 1968).

Reagents and materials

D-[^{14}C]Glucose 6-phosphate was chromatographed on Whatman no. 4 paper with 2-methoxyethanol-ethylmethylketone-aq. 1M-NH₃ (7:2:3, by vol.) (Mortimer, 1952).

Phosphoglucomutase from *M. lysodeikticus* was isolated from 25 g of spray-dried cells (Miles Laboratories Inc., Elkhart, Ind., U.S.A.) by the method of Hanabusa *et al.* (1966). It was stored in a small volume of 0.05M-Tris-acetate buffer, pH 7.0, at 4°C. The enzyme (specific activity 50–100 units/mg) had negligible phosphatase activity towards glucose 1-phosphate, glucose 6-phosphate and glucose 1,6-diphosphate; and it did not exhibit any phosphoglucose isomerase activity.

Methods

Total protein in the enzyme solution was determined from the E_{278} assuming $\epsilon_{1\text{cm}}^{1\%} = 10$.

To determine [^{14}C]glucose 1,6-diphosphate and glucose 1,6-[^{32}P]diphosphate, samples (0.1 ml) were added to 0.5 ml of ethanol (to inactivate the enzyme) and 0.4 ml of the mixture was applied to DEAE-cellulose chromatography paper (Whatman DE 81). Descending chromatography was carried out for 6h in 0.12M-ammonium formate, pH 6, and 1 cm strips were assayed for radioactivity by scintillation counting in 15 ml of Bray's (1960) solution.

Induced-transport tests. Experimental details of the induced-transport tests, which were carried out essentially as described in Britton & Clarke (1968), are given in the legends of Figs. 1–3.

Comparison of glucose and phosphate fluxes at chemical equilibrium. An appropriate quantity of the enzyme was added to a mixture of glucose 1-phosphate and glucose 1,6-diphosphate in 4.95 ml of

buffer (Tris, 36 mM adjusted to pH 8.3 with acetic acid, MgCl₂, 1.2 mM, cysteine, 5 mM) and the system was allowed to come to equilibrium at 30°C. A mixture of glucose 1-[^{32}P]phosphate and [^{14}C]glucose 6-phosphate was added in a small volume (0.05 ml). Two series of samples (0.1 ml) were taken to measure [^{14}C]glucose 1-phosphate and glucose 6-[^{32}P]phosphate radioactivity. [^{14}C]Glucose 1-phosphate radioactivity was measured as already described. All ^{32}P radioactivity was precipitated by the ZnSO₄-Ba(OH)₂ reagent. Glucose 6-[^{32}P]phosphate radioactivity was determined by following the procedure for glucose 1-[^{32}P]phosphate and assaying the radioactivity in the supernatant (Britton & Clarke, 1968). Glucose 6-[^{32}P]phosphate radioactivity was counted in the presence of [^{14}C]glucose 1-phosphate by appropriate setting of the discriminator of the scintillation counter.

Fluxes were calculated as described in Britton & Clarke (1968). No allowance was made for the appearance of radioactivity in glucose 1,6-diphosphate. The effect of this simplification must be small since the concentration of glucose 1,6-diphosphate was only about 5% of the glucose 1-phosphate concentration.

Comparison of maximum velocities in the forward and reverse directions. Solutions containing glucose 1,6-diphosphate and glucose 1-phosphate or glucose 6-phosphate were incubated in freshly prepared buffer (Tris-acetate 36 mM, pH 8.3, MgCl₂, 1.2 mM, cysteine, 5 mM) at 30°C. The same volume of enzyme was then added to each solution and samples (0.025 ml for the forward reaction, 0.2 ml for the backward reaction) were removed at intervals and the glucose 1-phosphate concentration was determined. The initial rate of conversion of glucose 1-phosphate into glucose 6-phosphate was determined by measuring the slope of the plot of glucose 1-phosphate concentration against time for the first 20–30% of the reaction. The initial rate of conversion of glucose 6-phosphate into glucose 1-phosphate was determined by extrapolating the linear unbiased plot of rate of formation of glucose 1-phosphate against time to zero time (Ray & Roscelli, 1964*b*).

Transfer of ^{14}C label from glucose 6-phosphate to glucose 1-phosphate and glucose 1,6-diphosphate at equilibrium. [^{14}C]Glucose 6-phosphate (approx. 4 μCi) in 50 μl was added to 4.95 ml of a solution of glucose 1-phosphate (0.1 mM), glucose 6-phosphate (1.72 mM) and glucose 1,6-diphosphate (0.1 mM) dissolved in freshly prepared buffer (Tris-acetate, 36 mM, pH 8.3, MgCl₂, 1.2 mM, and cysteine, 4.0 mM), containing 10 μl of the enzyme solution. Samples (100 μl) were withdrawn at intervals to determine [^{14}C]glucose 1,6-diphosphate. A second set of samples (100 μl) were taken to determine [^{14}C]glucose 1-phosphate. The fluxes were calculated as described by Britton & Clarke (1968).

Results

All experiments were carried out at 30°C in the Tris-acetate buffer, pH 8.3. Glucose 1,6-diphosphate was present at a concentration about four times greater than its K_m (approx. 0.1 μM) (Hanabusa *et al.*, 1966).

Induced-transport experiments with ^{32}P -labelled substrates at low substrate concentrations

In the experiment illustrated in Fig. 1 glucose 1- ^{32}P phosphate was incubated with the enzyme until equilibrium was reached and then unlabelled glucose

Fig. 1. Induced-transport test with ^{32}P -labelled substrates at low substrate concentrations showing co-transport

Glucose 1- ^{32}P phosphate was incubated at 30°C with phosphoglucomutase from *M. lysodeikticus* in Tris-acetate buffer, pH 8.3 (Tris-acetate, 36 mM, MgCl_2 , 1.2 mM, cysteine, 5 mM) in the presence of glucose 1,6-diphosphate, until equilibrium was reached. Samples were then taken from the reaction mixture and at zero time non-radioactive glucose 1-phosphate and a small quantity of glucose 1,6-diphosphate were added in a volume of 0.4 ml to 4.60 ml of the mixture. ●, Concn. of glucose 1-phosphate; ○, radioactivity in glucose 1- ^{32}P phosphate (d.p.m./ml), corrected for dilution caused by addition of non-radioactive glucose 1-phosphate. The continuous lines 1A, 1B and 1C are the theoretical curves for ^{32}P -labelled substrates for Schemes 1(a), 1(b) and 1(c) respectively assuming that any isomerization of the enzyme or phosphoenzyme is rapid ($\alpha = 0$). These theoretical lines were calculated by the method of Britton & Clarke (1968) and do not depend on rate constants. The broken line represents the concentration of glucose 1-phosphate. Concentration of hexose phosphates before addition of glucose 1-phosphate and glucose 1,6-diphosphate were glucose 1-phosphate, 8.2 μM , glucose 6-phosphate, 142 μM , glucose 1,6-diphosphate, 0.44 μM . Concentrations immediately after addition were glucose 1-phosphate, 158 μM , glucose 6-phosphate, 131 μM , glucose 1,6-diphosphate, 0.44 μM . Concentration of enzyme was about 3×10^{-2} $\mu\text{g/ml}$.

1-phosphate was added. During the reaction the radioactivity in the glucose 1-phosphate (total radioactivity not specific radioactivity) fell to a minimum of 42.5% and then returned to its original value. A second experiment gave a similar result with a minimum of about 46%. Since the transient fall in radioactivity in the glucose 1-phosphate must have been associated with a rise in the radioactivity in the glucose 6-phosphate, there was co-transport of the ^{32}P . Theoretical induced-transport curves (Fig. 1) were calculated with the assumption that any isomerization of the free enzyme is rapid (eqn. 2). They depend only on the concentrations of substrates and the equilibrium constant. The data closely fit the curve for Scheme 1(a) and differ widely from the curves for the other Schemes 1(b) and 1(c). If the isomerization of the free enzyme should be rate-limiting the theoretical curves are affected by the term α (eqn. 3). The theoretical amounts of co-transport shown by Schemes 1(a) and 1(b) are decreased and Scheme 1(c) should show counter-transport. In the limit when the isomerization is very slow the amount of co-transport shown by Scheme 1(a) becomes zero and that shown by Scheme 1(b) should approach that of Scheme 1(a) in the absence of a rate-limiting isomerization. The pattern for Scheme 1(a) when the isomerization is fast and to which the data conform could therefore also be given by Scheme 1(b) if the isomerization of the enzyme were the major rate-limiting step. This ambiguity can be resolved by induced-transport experiments with ^{14}C -labelled substrates as described below.

When unlabelled glucose 1-phosphate was added in the above experiments, the specific radioactivity of the glucose 1-phosphate decreased markedly. Some transfer of radioactivity from glucose 1,6-diphosphate to glucose 1-phosphate could therefore have taken place which would have decreased the apparent amount of co-transport. As discussed later, however, any such transfer should have been small.

Induced-transport experiments with ^{14}C -labelled substrates

Induced-transport experiments with ^{14}C -labelled substrates (Fig. 2) were carried out under conditions similar to those just described for ^{32}P -labelled substrates. In these experiments the radioactivity in the glucose 1-phosphate remained virtually constant over the whole period apart from a slight upward trend. Correspondingly the radioactivity in the glucose 6-phosphate must have also remained constant so that there was essentially no induced transport.

If it is assumed that any isomerization of the free enzyme (eqn. 2) is very rapid this is the expected result for Schemes 1(a) and 1(c) whereas Scheme 1(b) should give marked co-transport (see theoretical curves 1A, C and 1B of Fig. 2). If any isomerization of the

Fig. 2. Induced-transport test at low substrate concentrations with ^{14}C -labelled substrates showing absence of induced transport

$[^{14}\text{C}]$ Glucose 6-phosphate was incubated at 30°C with phosphoglucumutase from *M. lysodeikticus* in Tris-acetate buffer, pH 8.3, in the presence of glucose 1,6-diphosphate until equilibrium was reached. Samples were then taken from the reaction mixture and at zero time non-radioactive glucose 1-phosphate and a small quantity of glucose 1,6-diphosphate were added. For concentrations and other conditions see the legend to Fig. 1. ●, Concn. of glucose 1-phosphate; ○, $[^{14}\text{C}]$ glucose 1-phosphate radioactivity (d.p.m./ml) corrected for dilution caused by addition of glucose 1-phosphate. The averaged results of two experiments are shown. The continuous lines 1A, C and 1B are theoretical curves for Schemes 1(a), 1(c) and 1(b) respectively, assuming that any isomerization of the enzyme or phosphoenzyme is rapid ($\alpha = 0$). The curves do not depend upon rate constants. Curve 1'B was calculated for Scheme 1(b) with a rate-limiting isomerization of the enzyme ($\alpha = 500\text{mm}^{-1}$). All curves were calculated by the method of Britton & Clarke (1968). The broken line represents the concentration of glucose 1-phosphate.

free enzyme should be rate-limiting, the theoretical curves are affected by the rate constants through the term α . Schemes 1(a) and 1(c) should then show counter-transport, and with Scheme 1(b) the amount of co-transport should be decreased eventually to zero, when the isomerization of the free enzyme is much slower than the other steps in the reaction. Therefore, as with the induced-transport experiments with ^{32}P -labelled substrates, the experimental result could also be explained by Scheme 1(b) if the isomerization of the free enzyme (eqn. 2) were a very slow step. However, the theoretical curve 1'B (Fig. 2) is drawn for a value of α of 500mm^{-1} and it is clear that any co-transport that may have occurred must have been less than indicated by this line. As shown in the Appendix, this value of α would require that the K_m for glucose 1-phosphate should be over 20 times less

than the measured value (0.13 mM; Hanabusa *et al.*, 1966). To obtain a theoretical curve showing less co-transport would require a larger value for α and a correspondingly smaller maximum permissible value for the K_m . Scheme 1(b) therefore is excluded since α cannot be large enough to obtain a satisfactory fit to the data.

The slight upward trend in the radioactivity in the glucose 1-phosphate was probably due to equilibrium not being fully attained during the initial equilibration rather than due to phosphatase activity. Thus there was very little further increase over 8 h after the experimental period and the final value for the radioactivity in the glucose 1-phosphate corresponded well to that expected from the equilibrium constant for the reaction (1/17.2).

Transfer of radioactivity from glucose 1,6-diphosphate to glucose 1-phosphate

As discussed in relation to the induced-transport experiments with ^{32}P -labelled substrates, when unlabelled glucose 1-phosphate is added in the induced-transport test the specific radioactivity of the glucose 1-phosphate falls by a large factor and radioactivity may be transferred from glucose 1,6-diphosphate to glucose 1-phosphate. To estimate the magnitude of this phenomenon an experiment similar to that shown in Fig. 2 was carried out but the samples taken from the reaction mixture were chromatographed to separate glucose 1,6-diphosphate. The initial radioactivity in the glucose 1,6-diphosphate was about 4.2% of that in the glucose 1-phosphate. This corresponded reasonably well to isotopic equilibrium since the portion of glucose 1,6-diphosphate in the reaction mixture was 4.6% of the amount of glucose 1-phosphate. At 15, 47, 105 and 200 min after the addition of the unlabelled glucose 1-phosphate the radioactivity had fallen to 3.6, 2.8, 2.5 and 2.0% respectively. The last value again corresponded to isotopic equilibrium. From these data the contribution of ^{14}C radioactivity in glucose 1,6-diphosphate to glucose 1-phosphate and glucose 6-phosphate at 50 min was only about 2% and it is probable that much of this radioactivity entered the glucose 6-phosphate. Thus the contribution from glucose 1,6-diphosphate to glucose 1-phosphate in the induced-transport experiments with ^{14}C -labelled substrates must have been very small.

In the induced-transport experiments with ^{32}P -labelled substrates only the phosphate in the 1-position of the glucose 1,6-diphosphate may be expected to contribute substantially to radioactivity in the substrate (Britton & Clarke, 1968) and most of the phosphate so transferred will enter glucose 6-phosphate because of the co-transport of phosphate. Since the method of determining the ^{32}P in the glucose 1-phosphate also includes radioactivity in the 1-position of

glucose 1,6-diphosphate the data from the ^{14}C experiment indicates that the degree of co-transport in the ^{32}P experiments was overestimated by about 2%.

Induced-transport tests at intermediate substrate concentrations

Induced-transport tests with ^{32}P - and ^{14}C -labelled substrates were also carried out at substrate concentrations of the order of 1 mM. The experimental conditions were those given in the legend to Fig. 2, and the concentrations of hexose phosphates were, before addition of glucose 1-phosphate, glucose 1-phosphate, 0.044 mM, glucose 6-phosphate, 0.75 mM and glucose 1,6-diphosphate, 1.33 μM , and after addition of unlabelled glucose 1-phosphate and glucose 1,6-diphosphate, glucose 1-phosphate, 0.84 mM, glucose 6-phosphate, 0.696 mM and glucose 1,6-diphosphate, 1.33 μM . In two experiments with ^{32}P -labelled substrates the radioactivity in the glucose 1-phosphate fell to about

enzyme correspondingly more rapid. Taking values for the $V_{\max}$ and K_m with glucose 1-phosphate as substrate of 938 s^{-1} and 0.13 mM respectively (Hanabusa *et al.*, 1966) it follows (see the Appendix) that $(1/k_{+3}) + (1/k_{-3}) < 0.63 \times 10^{-6}\text{ s}$, or if $k_{+3} = k_{-3}$, $k_{+3} > 3.2 \times 10^6\text{ s}^{-1}$.

In the above, transfer of radioactivity from glucose 1,6-diphosphate to glucose 1-phosphate was ignored. As discussed for the previous experiments any such transfer is likely to have contributed less than 2% to the radioactivity already present in the glucose 1-phosphate and in any case such transfer would have caused a transient rise in the radioactivity in the glucose 1-phosphate.

Comparison of fluxes of glucose and phosphate at chemical equilibrium

For Scheme 1(a) at chemical equilibrium (Britton & Clarke, 1968):

$$\frac{\text{Flux of glucose from glucose 1-phosphate to glucose 6-phosphate}}{\text{Flux of phosphate from glucose 1-phosphate to glucose 6-phosphate}} = 2 + \alpha \quad (4)$$

and for Scheme 1(b)

$$\frac{\text{Flux of glucose from glucose 1-phosphate to glucose 6-phosphate}}{\text{Flux of phosphate from glucose 1-phosphate to glucose 6-phosphate}} = \frac{3 + 2\alpha}{2 + \alpha} \quad (5)$$

46 and 45% of the initial values which is in reasonable agreement with the minimum of 40.1% required by Scheme 1(a) in the absence of a rate-limiting isomerization of the enzyme. With ^{14}C -labelled substrate, there was no induced transport as required again by Scheme 1(a) in the absence of a rate-limiting isomerization of the phosphoenzyme.

Induced-transport tests at high substrate concentrations

The experiments described so far are entirely compatible with Scheme 1(a) provided that any isomerization of the enzyme is rapid. The effect of a rate-limiting isomerization upon the induced-transport test, however, becomes more prominent at high substrate concentrations. Induced-transport experiments were therefore carried out with ^{14}C -labelled substrates at a concentration in the order of 25 mM (Fig. 3). Again essentially no induced transport was found. A rate-limiting isomerization of the phosphoenzyme corresponding to a value of α of 11.5 M^{-1} would have led to a transient rise of 10% in the radioactivity in the glucose 1-phosphate after the addition of unlabelled glucose 1-phosphate. The results appear to exclude this possibility and therefore α must be larger than this value and the isomerization of the phospho-

Fig. 3. Induced-transport test at high substrate concentrations with ^{14}C -labelled substrates

For symbols and experimental procedure see the legend to Fig. 2. Concentrations of hexose phosphates before addition of non-radioactive glucose 1-phosphate and glucose 1,6-diphosphate were glucose 1-phosphate, 1.37 mM, glucose 6-phosphate, 23.6 mM, glucose 1,6-diphosphate, 0.10 mM. Concentrations immediately after addition were glucose 1-phosphate, 26.3 mM, glucose 6-phosphate, 21.7 mM, glucose 1,6-diphosphate, 0.10 mM. The horizontal line is the theoretical curve for Scheme 1(a) or 1(c) assuming that any isomerization of the phosphoenzyme is rapid (Britton & Clarke, 1968). Concentration of enzyme was about $0.3\text{ }\mu\text{g/ml}$.

Table 1. Fluxes of glucose and phosphate at chemical equilibrium determined by the use of ^{14}C - and ^{32}P -labelled substrates

Measurements were made in buffer (Tris-acetate, 36mM, MgCl_2 , 1.2mM, cysteine, 5mM), pH 8.3, at 30°C. Concentration of hexose phosphates were: Expt. 1, glucose 1-phosphate, 44 μM , glucose 6-phosphate, 756 μM , glucose 1,6-diphosphate, 1.33 μM ; Expt. 2: glucose 1-phosphate, 8.24 μM , glucose 6-phosphate, 141.8 μM , glucose 1,6-diphosphate, 0.44 μM .

Expt. no.	Concn. of enzyme ($\mu\text{g/ml}$)	Glucose flux ($\mu\text{mol/min per } \mu\text{g of enzyme}$)	Phosphate flux ($\mu\text{mol/min per } \mu\text{g of enzyme}$)	Ratio of glucose flux/phosphate flux	
				Ratio	Mean
1	0.82	4.09	2.08	2.00	2.03
		1.04	0.99	2.06	
2	0.16	8.57	4.36	1.97	1.95
		7.30	3.80	1.92	

The term α is defined by eqn. (3). For Scheme 1(c) the ratio should be unity. A series of such equilibrium measurements over a fivefold range of substrate gave a ratio close to 2 (Table 1). The data therefore again support Scheme 1(a) with a rapid isomerization of the enzyme although the same ratio would be given by Scheme 1(b) if α were large.

Ratio of maximum initial forward and reverse velocities

To determine the maximum permissible value for the K_m for any particular value of α , the ratio of maximum forward to maximum reverse velocity must be known (see the Appendix). Paired measurements of the initial rates in the two directions were made with solutions containing glucose 1,6-diphosphate (12 μM) and glucose 1-phosphate or glucose 6-phosphate (4mM). From four experiments a ratio V_F/V_R of 2.1 was obtained (range 2.0–2.4) with V_F and V_R referring to the velocities with glucose 1-phosphate and glucose 6-phosphate as substrate respectively. The glucose 1,6-diphosphate concentration in these experiments was approx. 80 times its K_m value (Hanabusa *et al.*, 1966) to minimize substrate inhibition, and the glucose 1-phosphate was approx. 30 times its K_m value. However, the glucose 6-phosphate was only approx. four times its K_m value (Hanabusa *et al.*, 1966) and V_R must have been underestimated to some extent. The effect of this on the maximum permissible value for the K_m for glucose 1-phosphate (see the Appendix), however, will be small and in any case the error will lead to an overestimate of the true value.

Exchange of ^{14}C between substrates and glucose 1,6-diphosphate

The enzyme-substrate complex of the phosphoglucomutase from rabbit muscle dissociates only very infrequently into glucose 1,6-diphosphate and the dephosphoenzyme (Ray & Roscelli, 1964a; Hashimoto & Handler, 1966). To examine the frequency of dissociation of the enzyme-substrate complex of the

Table 2. Fluxes of ^{14}C from glucose 6-phosphate to glucose 1-phosphate and glucose 1,6-diphosphate at equilibrium at pH 8.3 and 30°C

Concentration of hexose phosphates were glucose 6-phosphate, 1.72mM, glucose 1-phosphate, 0.10mM, glucose 1,6-diphosphate, 0.10mM. k_1 is the rate of accumulation of ^{14}C in glucose 1-phosphate and k_2 is the rate of accumulation of ^{14}C in glucose 1,6-diphosphate. The experiments were carried out in Tris-acetate buffer (Tris-acetate, 36mM, pH 8.3, MgCl_2 , 1.2mM, cysteine, 5mM).

Expt. no.	k_1 ($\mu\text{mol/min}$)	k_2 ($\mu\text{mol/min}$)	k_1/k_2
1	0.213	0.0232	9.2
2	0.287	0.0244	11.8

enzyme from *M. lysodeikticus*, [^{14}C]glucose 6-phosphate was added to a solution of an equilibrium mixture of glucose 1-phosphate and glucose 6-phosphate, glucose 1,6-diphosphate and enzyme (Table 2). The rate of entry of the label into the glucose 1,6-diphosphate was over ten times slower than the rate of entry into glucose 1-phosphate indicating that dissociation occurs in less than one in ten turnovers. As with the rabbit muscle enzyme therefore the enzyme-substrate complex dissociates relatively infrequently into glucose 1,6-diphosphate and dephosphoenzyme.

Discussion

The present data provide strong evidence that phosphoglucomutase from *M. lysodeikticus* possesses a phosphoenzyme mechanism. Thus, both the induced-transport data and the exchange at chemical equilibrium completely exclude any intramolecular mechanism (Scheme 1c); and the intermolecular or sequential mechanism (Scheme 1b) would only have been compatible with the data if an isomerization of the enzyme (eqn. 2) were the major rate-limiting step and if the K_m for the enzyme had been at least 20 times less than the measured value. In contrast, all of

the data are entirely in accord with a phosphoenzyme mechanism provided that any isomerization step has a rate constant for isomerization in excess of about $3 \times 10^6 \text{ s}^{-1}$. As discussed by Britton & Clarke (1968) none of these conclusions should be substantially affected by non-ideal behaviour of the solution or isotope effects.

The present conclusions about the mechanism do not unequivocally establish the chemical nature of the phosphoenzyme and it is possible that this may be an enzyme-glucose 1,6-diphosphate complex in which one of the phosphates of the glucose 1,6-diphosphate plays the role of the phosphate in the phosphoenzyme, rather than a true phosphoenzyme. However, after incubation with ^{32}P - and ^{14}C -labelled glucose 1,6-diphosphate and separation by gel filtration the enzyme was labelled with ^{32}P (1 mol/mol of enzyme) but not with ^{14}C (Birch *et al.*, 1972; Clarke *et al.*, 1974). In further work (Milstein *et al.*, 1973) the phosphate was shown to be attached to serine and the sequence around the serine, Thr-Ala-Ser(P)-His-Asp, was found to be the same as in the rabbit muscle enzyme (Milstein & Sanger, 1961). Thus a true phosphoenzyme may be involved.

The converging initial-velocity plots obtained by Hanabusa *et al.* (1966) when the reciprocal of the initial velocity was plotted against the reciprocal of the glucose 1-phosphate concentration at different glucose 1,6-diphosphate concentrations and vice versa appear to be at variance with the present evidence for a phosphoenzyme mechanism. Such a pattern could arise if the 'phosphoenzyme' was an enzyme-glucose 1,6-diphosphate complex but as discussed above a true phosphoenzyme appears the more probable intermediate. Alternatively a converging-line pattern could be obtained if the phosphoenzyme were unstable (Britton & Clarke, 1968). However, Hanabusa

et al. (1966) found that the enzyme had no glucose 1,6-diphosphate activity and the phosphoenzyme isolated by Birch *et al.* (1972) had a half-life of 46 h. Thus neither of the two possibilities appear likely. However, in the experiments of Hanabusa *et al.* (1966) relatively small errors in the determinations of the initial velocities could have led to the convergence observed. Such errors could arise at low glucose 1-phosphate and glucose 1,6-diphosphate concentrations, since the effective K_m for glucose 6-phosphate will be very small and appreciable product inhibition may have occurred. Further, their results were complicated by substrate and cofactor inhibition at high substrate and cofactor concentrations.

The present kinetic experiments suggest strongly that there is no essential difference between the mechanism of the phosphoglucosyltransferases from rabbit muscle and from *M. lysodeikticus*. Since in related work mentioned above (Birch *et al.*, 1972; Clarke *et al.*, 1974; Milstein *et al.*, 1973) the phosphate of the phosphoenzyme has been shown to be covalently bound to a serine located in a similar amino acid sequence to that found in the rabbit muscle enzyme, it seems likely that both enzymes have a common evolutionary origin. In both cases the rate constants for isomerization for the phosphoenzyme are very high. As pointed out by Ray & Roscelli (1964b) such high values are not compatible with a phosphoenzyme mechanism as depicted in Scheme 1(a), because this scheme involves a migration of the phosphate of the phosphoenzyme. We propose therefore the alternative mechanism illustrated in Scheme 2 in which there is no formal rearrangement of the phosphoenzyme. Instead the enzyme-substrate complex rearranges so that either the 1- or the 6-position of the glucose 1,6-diphosphate is attacked by the active serine of the enzyme. This mechanism, if the

Scheme 2. Modified phosphoenzyme mechanism for the phosphoglucosyltransferase reaction to account for the very rapid phosphoenzyme isomerization

E represents the enzyme and E-P the phosphoenzyme. The dashed arrows indicate slow reactions.

phosphoenzyme should be unstable, can also be regarded as that of a glucose 1,6-diphosphatase which is able to attack either of the phosphates of the substrate. Thus the phosphoglucomutase enzymes may have developed from a glucose 1,6-diphosphatase. The dissociation of the enzyme-substrate complex into glucose 1,6-diphosphatase and dephosphoenzyme is shown as a slow reaction (Scheme 2) since the present findings indicate that dissociation occurs in only about one in ten of turnovers and the frequency of dissociation with rabbit muscle enzyme is even less (Ray & Roscelli, 1964a; Hashimoto & Handler, 1966). A similar mechanism to that in Scheme 2 has been proposed for the 2,3-diphosphoglycerate-dependent phosphoglycerate mutases (Britton & Clarke, 1972a) but as pointed out with these enzymes, the phosphoglycerate mutases and the phosphoglucomutases may not be related but may represent parallel evolution, since the phosphoglycerate mutases do not require Mg^{2+} for activity (Rodwell *et al.*, 1957) and the phosphate of the phosphoenzymes is probably attached to histidine (Rose, 1970, 1971; Britton *et al.*, 1972).

We thank Mr. M. Birch for skilled technical assistance and the S.R.C. for financial support.

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APPENDIX

Maximum permissible value of the K_m

From Britton & Clarke (1968):

$$K_m < (1 + V_F/V_R)/\alpha \quad (A1)$$

where K_m is the Michaelis constant with glucose 1-phosphate as a substrate, V_F is the maximum velocity/mol of enzyme with glucose 1-phosphate as a substrate and V_R is the maximum velocity/mol of enzyme with glucose 6-phosphate as a substrate. From the main paper $V_F/V_R = 2.1$ and $\alpha = 500 \text{ mm}^{-1}$, and substituting these values in eqn. (A1), $K_m < 6.2 \mu\text{M}$.

Rate constants for phosphoenzyme isomerization

From Britton (1973), provided that $\alpha[S_\infty] \ll 1$

$$[S^*]/[S_0^*] = 1 + \alpha([S_0] - [S_\infty])/e \quad (A2)$$

where $[S^*]$ = radioactivity in glucose 1-phosphate, $[S_0^*]$ = initial radioactivity in glucose 1-phosphate, $[S_0]$ = initial concentration of glucose 1-phosphate, $[S_\infty]$ = final concentration of glucose 1-phosphate. α is defined by eqn. (2) of the main paper.

In Fig. 4 of the main paper, assuming an equilibrium constant of 1/17.2 (Britton & Clarke, 1968), $[S_\infty] = 2.64 \text{ mM}$ and $[S_0] = 26.3 \text{ mM}$. Further, for a 10% rise in radioactivity $[S^*]/[S_0^*] = 1.1$, and substituting in eqn. (A2) gives $\alpha = 1.15 \times 10^{-2} \text{ mm}^{-1}$. From Britton & Clarke (1968):

$$\alpha K_m/V_F = (1/k_{+3}) + (1/k_{-3}) \quad (A3)$$

From Hanabusa *et al.* (1966), $K_m = 0.13 \text{ mM}$ and $V_F = 938 \text{ s}^{-1}$. Substituting these values in eqn. (A3):

$$(1/k_{+3}) + (1/k_{-3}) = 0.63 \times 10^{-6} \text{ s}$$

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