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THE PHOSPHOGLUCOMUTASE REACTION: INVESTIGATION OF ENZYME DEPHOSPHORYLATION DURING ITS REACTION WITH SUBSTRATE

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SUMMARY

1. The degree to which phosphoglucomutase (α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) is dephosphorylated when the phosphoenzyme is incubated with various levels of ^{32}P-labeled glucos e6-phosphate, was found to be considerably less than that predicted on the basis of previous reports concerning the equilibria involved in the enzyme mechanism. Measurements of the specific radioactivity of the protein precipitated by the addition of trichloroacetic acid to the incubation mixtures were used in evaluating the extent of dephosphorylation of the enzyme, and the results were comparable to those obtained by chromatographic separation of phosphoenzyme and dephosphoenzyme. That the relatively high specific radioactivity of the precipitated enzyme truly represented phosphoenzyme remaining after incubation, and not absorbed ^{32}P-labeled substrate, was confirmed (a) by repeated solution and precipitation of the enzyme and (b) by substituting ^{14}C-labeled glucose 6-phosphate for the ^{32}P-labeled compound, in which case essentially no radioactivity was associated with the precipitated protein.

2. Analysis of the non-protein portion of incubation mixtures revealed that glucose 1,6-diphosphate was present in concentrations much lower than would be consistent with the previously reported equilibria comprising the phosphoglucomutase reaction. Investigation of the transfer of radioactive phosphate from ^{32}P-labeled phosphoenzyme during short-term incubations with unlabeled glucose 6-phosphate resulted in labeling patterns which were consistent with the suggestion that phosphoglucomutase catalyzes the reversible transfer of phosphate from glucose 6-phosphate to form glucose 1-phosphate without the obligatory formation of free glucose 1,6-diphosphate.

Abbreviations: *E* and *EP*, dephospho- and phospho-forms of the enzyme phosphoglucomutase, respectively. The subscript, o (e.g., EP_o), when used, refers to concentrations of the designated species present initially, prior to incubation.

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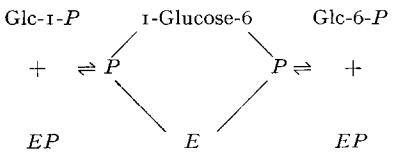
INTRODUCTION

Phosphoglucomutase (α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) catalyzes the reversible transfer of phosphate between Glc-1-*P* and Glc-6-*P*. In 1949 an investigation of the mechanism based on ^{32}P-exchange studies prompted JAGANNATHAN AND LUCK[1] to propose the participation of a phosphorylated enzyme species in the reaction:



(1)

where *EP* and *E* are the phosphorylated and dephosphorylated forms of the enzyme, respectively.

Following the identification of glucose 1,6-diphosphate and the demonstration that this compound was required for enzyme activity[2,20], NAJJAR AND PULLMAN[3] suggested the two-step mechanism:

$$\text{Glc-1-}P + EP \rightleftharpoons \text{Glc-1,6-}P_2 + E \tag{2}$$

$$\text{Glc-1,6-}P_2 + E \rightleftharpoons \text{Glc-6-}P + EP \tag{3}$$

The first shadow was cast on this mechanistic interpretation by the kinetic studies reported by BODANSKY[4]. Based on these studies CLELAND[5] suggested that the apparent kinetics of the reaction could best be interpreted as a one-step sequential pathway:

$$E + \text{Glc-1-}P + \text{Glc-1,6-}P_2 \rightleftharpoons E + \text{Glc-6-}P + \text{Glc-1,6-}P_2 \tag{4}$$

The recent study by RAY AND ROSCELLI[6] in which they re-examined the substrate–velocity relationship, substrate inhibition, and the role of glucose 1,6-diphosphate has prompted these authors to reject CLELAND's suggestion and to favor a modified ping-pong pathway. RAY AND ROSCELLI[6] suggest that Glc-1,6-P_2 is an abortive product of the enzyme–*P*–substrate complex and not an obligatory intermediate. These authors propose that the function of the diphosphate is to prevent a depletion of *EP*, the active enzyme species, by phosphorylating the dephosphoenzyme, which is one of the abortive products.

$$EP + \text{Glc-1-}P \rightleftharpoons \begin{bmatrix} EP\cdot\text{Glc-1-}P \\ E\cdot\text{Glc-1,6-}P_2 \\ EP\cdot\text{Glc-6-}P \end{bmatrix} \rightleftharpoons EP + \text{Glc-6-}P \qquad \Updownarrow \qquad \text{Glc-1,6-}P_2 + E \tag{5}$$

Concurrent studies in our laboratory have been focused on dephosphorylation of the phosphoenzyme, *EP*, examination of the equilibrium constant for Reaction 3 (see ref. 7), and the transfer of phosphate from the *EP* with ^{32}P-tracer studies. The findings, which are presented in this report, appear to be in accord with the suggestions of RAY AND ROSCELLI[6].

METHODS

Enzyme preparation

The enzyme was prepared from rabbit skeletal muscle as described previously[8]. ^{32}P-labeled enzyme was prepared as described by RAY AND KOSHLAND[9,10].

Protein concentrations were estimated by absorbance at 278 mμ employing 7.7 as the absorbance of a 1% solution of the enzyme. Enzyme concentrations in terms of μM were determined employing a molecular weight of 62 000 (see ref. 11).

Enzyme assay

The enzymatic activity was assayed essentially as described by NAJJAR[12] except that the incubation mixture contained a buffer of histidine, 0.04 M, and Tris, 0.04 M, instead of cysteine.

Preparation of trichloroacetic acid-precipitated protein for counting

The protein was precipitated from the reaction mixture by the addition of trichloroacetic acid to a final concentration of 10%. This suspension was allowed to stand in an ice-bath for a minimum of 1 h and frequently overnight. After separating the precipitated protein by centrifuging for 20 min at top speed using a clinical centrifuge, the supernatant was removed and the precipitate resuspended in 5% trichloroacetic acid. After centrifuging, the 5% trichloroacetic acid wash was plated on an aluminum planchet for measurement of radioactivity. The wash procedure was continued until the radioactivity approached background. The trichloroacetic acid-precipitated protein was then dissolved in 90% formic acid. Occasionally, the protein was reprecipitated by the addition of excess trichloroacetic acid, washed, and finally redissolved in 90% formic acid. The entire formic acid–protein solution and two test-tube washes were plated on aluminum planchets and dried under an infrared lamp, and the ^{32}P associated with the protein was determined from the counting data obtained.

Analysis of phosphate components of the reaction mixtures

In order to analyze the ^{32}P-containing material present in the supernatant following enzyme incubation, the supernatant and the first 5% trichloroacetic acid wash were combined. The resulting solution was extracted three times with an equal volume of ether. Final traces of ether were removed by evaporation. An aliquot of this solution was plated and from it the total radioactivity of the solution was determined. The remainder of the solution was chromatographed at room temperature on a 14.5 cm $\times$ 0.5 cm column of Dowex 1-X8, 200–400 mesh, in the formate form. The phosphate compounds were eluted using first a linear gradient comprised of 250 ml of water and 250 ml of 1.0 M formic acid, and then a linear gradient of 250 ml of water and 250 ml 2 M ammonium formate. In this chromatographic system the glucose monophosphates are eluted as a single peak separated from inorganic phosphate by the formic acid gradient, whereas glucose 1,6-diphosphate is eluted in the ammonium formate gradient. The phosphate compounds were detected by plating and counting an aliquot of each fraction.

Analysis of the phosphate components

Total phosphate was determined by the method of BARTLETT[13], and inorganic phosphate was determined by the isobutanol–benzene extraction procedure of MARTIN AND DOTY[14]. An aliquot of the organic layer was plated and counted for $^{32}P_i$ determinations. Glucose 1-phosphate was determined as acid-labile phosphate by the same extraction procedure after a 10-min period of hydrolysis in 1.0 M HCl in a boiling-water bath. Glucose 6-phosphate was determined as the difference, *i.e.*, the total ^{32}P radioactivity *minus* the directly extractable inorganic *plus* acid-labile phosphate.

Preparation of glucose 6-[^{32}P]phosphate

Glucose 6-[^{32}P]phosphate was prepared with sucrose phosphorylase as described previously[10]. Following the isolation of the acid-stable phosphate from the preparative procedure the material was hydrolyzed to destroy any glucose 1-[^{32}P]phosphate and then chromatographed on Dowex 1-formate.

Ammonium sulfate precipitation of protein in reaction mixtures

Solid ammonium sulfate was added to the reaction mixture slowly, in the cold, to 70% saturation. The ammonium sulfate-precipitated material was allowed to stand in the cold overnight and the precipitate was collected by centrifugation at 20 000 rev./min.

RESULTS

During a series of studies in which it had been assumed that completely dephosphorylated enzyme had been prepared according to procedures previously reported[15], it became apparent that this assumption might not be justified. In order to investigate this possibility, phosphoglucomutase was incubated with glucose 6-[^{32}P]phosphate in ratios ranging from 1:25 to 1:90. A representative series of the results obtained in such experiments is presented in Table I. The results indicate that a considerable fraction of the enzyme is labeled with ^{32}P after trichloroacetic acid-precipitation following incubation at glucose 6-phosphate:enzyme ratios of 90 and 25.

To determine the effect of two successive incubations of the enzyme with excess glucose 6-[^{32}P]phosphate, the following experiment was performed. At the end of the first incubation an aliquot was precipitated with trichloroacetic acid and the ^{32}P-content of the trichloroacetic acid-precipitate was determined. To the remaining incubation mixture, solid ammonium sulfate was added to 70% saturation. After standing in the cold overnight, the ammonium sulfate precipitate was collected by centrifugation, was dialyzed, and was incubated with glucose 6-[^{32}P]phosphate (Glc-6-*P*:enzyme ratio of 83) a second time. After 20 min the protein was precipitated with trichloroacetic acid. The results (Expts. 3A and 3B, Table I) indicate that repeated incubation did not affect the extent to which ^{32}P was associated with the enzyme.

The possibility that a significant fraction of the ^{32}P associated with the trichloroacetic acid-precipitated enzyme might reflect substrate adsorption rather than covalently bound phosphate was examined by the following experiment. Two parallel incubations were performed (Expts. 4A and 4B, Table I) in which both were treated in an identical manner up to the point where the washed trichloroacetic acid-precipitated protein was to be dissolved. At this point, one precipitate, 4A, was dissolved in

TABLE I

FRACTION OF PHOSPHOGLUCOMUTASE LABELED WITH ^{32}P FOLLOWING 'DEPHOSPHORYLATION' INCUBATION

Incubations were performed in presence of 0.0015 M histidine–Tris buffer (pH 7.5), 0.0015 M $MgSO_4$ at 30° for 20 min with glucose 6-[^{32}P]phosphate at the molar ratios of glucose 6-[^{32}P]phosphate:enzyme indicated. Enzyme concentrations in experiments cited: (1) $2.7 \cdot 10^{-5}$ M; (2) $4.4 \cdot 10^{-5}$ M; (3A) $5.6 \cdot 10^{-5}$ M, (3B) $3.7 \cdot 10^{-5}$ M; (4 A and B) $7 \cdot 10^{-5}$ M; (5) $9.2 \cdot 10^{-5}$ M; (6A, B, and C) $4.7 \cdot 10^{-5}$ M; (7A and B) $8 \cdot 10^{-5}$ M. Following a 5-min period of thermal incubation, reactions were initiated by the addition of enzyme. The reactions were routinely halted by addition of 50% trichloroacetic acid to a final concentration of 10%, and protein precipitates prepared for radioactivity determinations as described in text. Variations to this procedure indicated in the table.

Experiment number	*Experimental variation from standard procedure described in legend*	*Glucose 6-[^{32}P]phosphate:enzyme ratio during incubation*	*Fraction of enzyme labeled with ^{32}P after incubation*	
			Experimental	*Theoretical*
1	None	90	0.80	<0.04
2	None	24.6	0.64	0.12
3 A	Aliquot precipitated with trichloroacetic acid after single incubation	64	0.63	0.05
3 B	$(NH_4)_2SO_4$ precipitate of 3A dialyzed and incubated with glucose 6-[^{32}P]-phosphate a second time	83	0.63	0.04
4A	None	42	0.59	0.08
4B	Trichloroacetic acid-precipitated protein dissolved in 0.1 M NaOH and reprecipitated with trichloroacetic acid	42	0.59	0.08
5	Aliquot precipitated with trichloroacetic acid; remainder with $(NH_4)_2SO_4$ and chromatographed	46	0.46 (0.59–0.48* by chromatography)	0.07
6A	10-min incubation	41	0.39	0.08
6B	40-min incubation	41	0.41	0.08
6C	60-min incubation	41	0.38	0.08
7A	None	86	0.80	0.04
7B	Enzyme incubated under activating conditions prior to glucose 6-[^{32}P]-phosphate	86	0.78	0.04

* See text.

90% formic acid and plated; the other precipitate, 4B, was dissolved in 0.1 M NaOH and then reprecipitated with trichloroacetic acid prior to being prepared for plating. If the ^{32}P associated with the protein consisted of adsorbed glucose 6-[^{32}P]phosphate as a result of the large excess of this reagent present at the initial precipitation, then the amount of ^{32}P associated with the enzyme would have been substantially reduced by the second procedure. As shown in Table I, the same quantity of ^{32}P was associated with the protein in both cases.

When the enzyme chromatographic procedure for the separation of phosphoenzyme and dephosphoenzyme had been refined[8], this additional analytical procedure was employed to confirm that the ^{32}P was covalently bound to the enzyme. At the end of the incubation period an aliquot was removed and the ^{32}P-labeled enzyme was determined by the trichloroacetic acid-precipitation technique. The remaining reac-

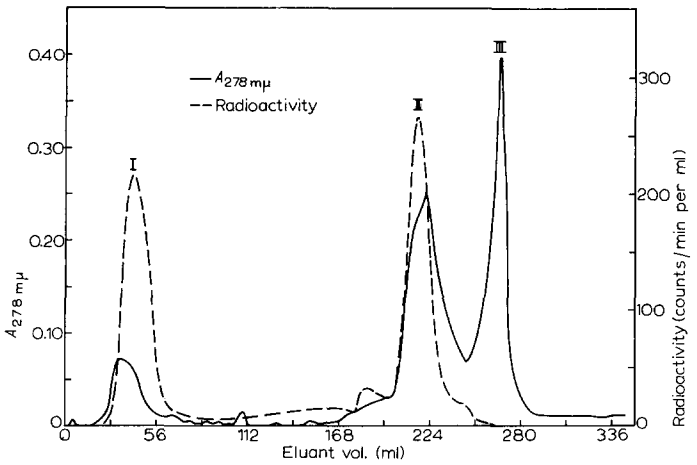


Fig. 1. Chromatography of phosphoglucomutase following incubation with glucose 6-[^{32}P]phosphate. Conditions: Expt. 5, Table I, containing 0.498 μmole enzyme, 20.9 μmoles glucose 6-[^{32}P]-phosphate in total volume of 5.44 ml, 0.0125 M in histidine–Tris (pH 7.5) and 0.0015 M in $MgSO_4$. Incubation period, 20 min at 30°. Aliquot precipitated with trichloroacetic acid, and remainder of protein precipitated with ammonium sulfate and prepared for chromatography. Protein applied to 60 cm × 1.5 cm column of CM-cellulose at a concentration of 2 mg/ml in 0.001 M sodium phosphate (pH 7) at 4°; eluted with linear gradient of sodium phosphate, 0.001 M to 0.10 M (pH 7.5), total volume 750 ml.

tion mixture was adjusted to 70% saturation with ammonium sulfate, and after standing in the cold overnight, the protein was collected by centrifugation and prepared for chromatography. The elution pattern obtained in this chromatography is shown in Fig. 1. If one considers Peaks I and II to be phosphoenzyme and Peak III dephosphoenzyme, then the fraction of phosphoenzyme calculated by chromatographic separation would be 0.58. However, if one considers that only Peak II is phosphoenzyme and Peak III dephosphoenzyme (which is more consistent with enzymatic activity data of the fractions) then the fraction of phosphoenzyme is 0.48, a result very similar to that obtained by analysis of the radioactivity associated with the trichloroacetic acid-precipitated protein (Expt. 5, Table I). It had been shown that rechromatography of the phosphoglucomutase emerging as the phosphoenzyme peak (Peak II) resulted in the elution of a single phosphoenzyme peak[8]; thus the chromatographic separation was not an artifact attributable to association–dissociation effects on the column. The results obtained in such experiments support the conclusion that phosphoenzyme is present following its incubation with glucose 6-phosphate in large excess, and in amounts considerably greater than that predicted on the basis of the equilibrium constant reported for the reaction shown in Eqn. 3 (see ref. 7).

In an essentially identical experiment, the ^{32}P:enzyme ratio was determined as a function of incubation time (Expt. 6, Table I). The fraction of phosphoenzyme present in the reaction mixture was found to be the same following 10, 40, and 60 min incubation.

It was also of interest to determine if pre-activated enzyme, as described by ROBINSON AND NAJJAR[16] would show any variation. In this case (Expts. 7A and 7B, Table I) two incubations were performed concurrently. One, 7A, was performed

according to the usual procedure, whereas in the second, 7B, the enzyme was first pre-incubated for 5 min at 30° in the presence of 0.0125 M histidine–Tris buffer (pH 7.5) and 0.0015 M $MgSO_4$. In the latter experiment the reaction was initiated by adding glucose 6-[^{32}P]phosphate. As shown in Table I, pre-incubation of the enzyme produced no observable change in the amount of phosphoenzyme resulting from incubation with glucose 6-phosphate.

In the series of experiments described thus far, phosphoglucomutase was incubated with a large excess of glucose 6-phosphate. When the ratio of Glc-6-*P*:enzyme was varied in the range of 8.0 to 0.12, similar conclusions were reached. The results obtained in these experiments are presented in Table IIA. Since the phosphate contributed by phosphoenzyme present at the beginning of incubation would significantly dilute the specific radioactivity of the total phosphate in the reaction mixtures, it was necessary to calculate the final specific radioactivity of the ^{32}P-labeled phosphate. Knowing the total radioactivity added in each incubation and the specific radioactivity of the glucose 6-[^{32}P]phosphate, it was possible to determine by successive approximation the ratio of phosphoenzyme to total enzyme prior to treatment with labeled Glc-6-*P*. Also the ratio of ^{32}P-labeled phosphoenzyme:total enzyme, predicted on the basis of an equilibrium constant of 3.76 for Eqn. 3 (see ref. 7), was calculated for each incubation. The assumption that the original phosphoenzyme concentration was equal to zero ($EP_0 = 0$) is obviously invalid as evidenced by the fraction of EP calculated from the experimental data for this case. At each of the other assumed EP_0 fractions, the ratio of ^{32}P:enzyme calculated from the experimental data appears to be independent of the ratio of glucose 6-[^{32}P]phosphate to enzyme concentration during the incubation. None of the experimentally determined results, at any assumed EP_0 fraction, follow the trend predicted on the basis of the NAJJAR mechanism.

TABLE IIA

FRACTION OF PHOSPHOGLUCOMUTASE LABELED WITH ^{32}P FOLLOWING INCUBATION WITH VARIOUS GLUCOSE 6-[^{32}P]PHOSPHATE CONCENTRATIONS

Incubations were performed with glucose 6-[^{32}P]phosphate in 0.0125 M histidine–Tris buffer (pH 7.5), 0.0015 M $MgSO_4$, total volume 0.5 ml, for 5 min at 30°. Reactions were terminated by addition of 0.12 ml of 50% trichloroacetic acid. Precipitates were collected and prepared for determination of radioactivity as described in the text. Enzyme concentrations: (1) 0.116 μmole/ml; (2) 0.122 μmole/ml; (3) 0.166 μmole/ml; (4) 0.098 μmole/ml; and (5) 0.167 μmole/ml. Specific radioactivity of phosphate following incubation was calculated for each assumed fraction of enzyme present as phosphoenzyme prior to incubation, EP_0/Enz. These values were employed in calculating the μmoles phosphate bound per μmole enzyme for each reaction.

Expt. No.	*Glucose 6-[^{32}P]phosphate:enzyme incubation ratio*	*Fraction of enzyme labeled with ^{32}P (μmoles ^{32}P/μmole enzyme)*					
		Assuming $K = 3.76$ and $E = Glc\text{-}1,6\text{-}P_2$		*Based on radioactivity of trichloroacetic acid-precipitated protein*			
		$\frac{EP_0}{Enz} = 0.6$	$\frac{EP_0}{Enz} = 1$	$\frac{EP_0}{Enz} = 0$	$\frac{EP_0}{Enz} = 0.6$	$\frac{EP_0}{Enz} = 0.8$	$\frac{EP_0}{Enz} = 1$
1	8.0	0.16	0.26	0.64	0.71	0.74	0.70
2	3.8	0.28	0.38	0.54	0.62	0.62	0.67
3	0.91	0.43	0.67	0.38	0.64	0.73	0.81
4	0.23	0.54	0.86	0.16	0.60	0.73	0.89
5	0.12	0.57	0.91	0.096	0.57	0.70	0.89

TABLE IIB

FRACTION OF PHOSPHOGLUCOMUTASE LABELED WITH ^{14}C FOLLOWING INCUBATION WITH [^{14}C]GLUCOSE 6-PHOSPHATE

Incubation conditions identical to those in Table IIA except substituting [^{14}C]glucose 6-phosphate for glucose 6-[^{32}P]phosphate. Enzyme concentration, both experiments, 0.086 μmole/ml.

Expt. No.	[^{14}C]*Glucose 6-phosphate: enzyme incubation ratio*	^{14}C/*enzyme**
1	11.9	0.021
2	19.2	0.023

* Based on radioactivity of trichloroacetic acid-precipitated protein.

In order to determine if part of the ^{32}P associated with the enzyme in these experiments resulted from glucose 6-[^{32}P]phosphate strongly bound to the protein (rather than *EP*, phosphoenzyme), phosphoglucomutase from the same enzyme preparation was incubated with ^{14}C-labeled glucose 6-phosphate under identical conditons. The results, presented in Table IIB, demonstrate that the quantity of Glc-6-*P* bound to the enzyme is minimal and certainly not sufficient to account for the high fraction of ^{32}P-labeled enzyme experimentally observed.

These results prompted a detailed study of the entire phosphoglucomutase reaction, which included quantitation of all the chemical species at equilibrium and calculation of the apparent equilibrium constant based on the data obtained. Three

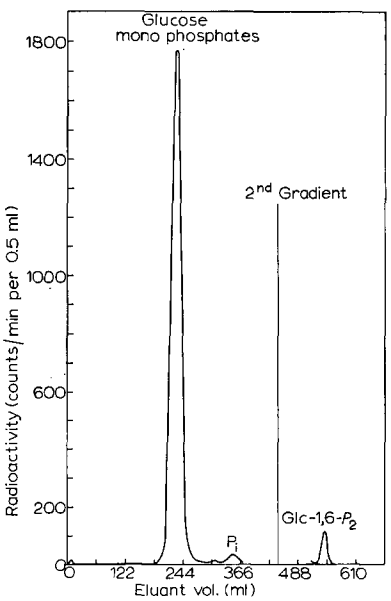


Fig. 2. Chromatography of trichloroacetic acid-supernatant from 10-min incubation of enzyme with glucose 6-[^{32}P]phosphate. Column = 14.5 cm × 0.5 cm, Dowex 1-X8, 200–400 mesh, in formate form, eluted by applying two successive linear gradients: (a) $H_2O \rightarrow 1$ M formic acid, total volume 500 ml; (b) $H_2O \rightarrow 2$ M ammonium formate, total volume 500 ml.

TABLE III

DISTRIBUTION OF ^{32}P-LABELED PHOSPHATE FOLLOWING INCUBATION OF PHOSPHOGLUCOMUTASE WITH GLUCOSE 6-[^{32}P]PHOSPHATE

Three separate reaction mixtures, each containing 0.2066 μmole phosphoglucomutase and 0.8735 μmole glucose 6-[^{32}P]phosphate in a total volume of 3.04 ml, 0.0125 M in histidine–Tris (pH 7.5) and 0.0015 M in $MgSO_4$, were incubated at 30° for 10, 40, and 180 min, respectively. Reactions were stopped at times indicated by addition of 0.76 ml of 50% trichloroacetic acid. Protein precipitates and supernatants were prepared as indicated in the text for determination of the ^{32}P/enzyme molar ratio and concentrations of Glc-6-*P*, Glc-1-*P*, P_i, and Glc-1,6-P_2. Two quantitations were made: (1) assuming that all the phosphoglucomutase was non-phosphorylated prior to incubation, EP_0/total enzyme = 0, thus employing the specific radioactivity of the initial glucose 6-[^{32}P]phosphate to quantitate the phosphate distribution, and (2) assuming the enzyme was phosphorylated prior to incubation, EP_0/total enzyme = 1, thus calculating the final specific radioactivity of phosphate by considering the isotopic dilution of the glucose 6-[^{32}P]phosphate by the phosphate present on the enzyme.

μmoles in 3.04 ml at time, t	*Assuming EP_0/total enzyme = 0*			*Assuming EP_0/total enzyme = 1*		
	10 min	*40 min*	*180 min*	*10 min*	*40 min*	*180 min*
Glc-6-*P*	0.8025	0.8062	0.7879	0.9922	0.9967	0.9742
Glc-1,6-P_2	0.0112	0.0104	0.0195	0.0138	0.0128	0.0242
^{32}P/enzyme, molar ratio	0.476	0.466	0.449	0.588	0.576	0.555
$EP_{(t)}$	0.0851	0.0875	0.0919	0.1215	0.1191	0.1147
$E_{(t)}$	0.1083	0.1103	0.1138	0.0983	0.0963	0.0928

identical reaction mixtures were prepared and incubations were performed under the conditions previously described. The three reactions were halted at 10, 40, and 180 min, successively, by the addition of trichloroacetic acid. The trichloroacetic acid supernatant from each reaction, including the first wash, was prepared for chromatography on Dowex 1- formate columns as described. In this manner glucose monophosphate, inorganic phosphate, and glucose 1,6-diphosphate were resolved. A typical chromatogram is illustrated in Fig. 2. As this chromatographic procedure does not separate the glucose monophosphates, Glc-1-*P* was distinguished from Glc-6-*P* by determining the fraction of acid-labile phosphate present in the isolated glucose monophosphate peak. The results are presented in Table III. Based on the experimental data, apparent equilibrium constants, K_{app}, were calculated assuming that all phosphate present had attained complete equilibrium within the designated incubation periods (Table IV). Examination of the results given in Tables III and IV reveals that both $K_{app(10)}$ (based on the radioactive species present after 10 min incubation) and $K_{app(40)}$ (40 min incubation) differ from $K_{app(180)}$ by a factor of approx. 2, reflecting a similar difference in the quantity of Glc-1,6-P_2 formed.

Since quantitation was determined by radioactivity and was thus based on the assumption that the ^{32}P was completely randomized among all the reacting phosphate species within each incubation period, a re-evaluation of this assumption seemed in order. Two possible sites of incomplete isotopic equilibration were considered, (a) the phosphate moiety of phosphoenzyme and (b) the two phosphate groups of glucose 1,6-diphosphate. The apparent equilibrium constants resulting from calculations based on each of these possibilities are given in Tables III and IV. It can be seen that $K_{app(t)}$ values for Condition a, incomplete equilibration of enzyme phosphate, are considerably greater than the equilibrium constant previously reported, and again $K_{app(10)}$ and

TABLE IV

APPARENT EQUILIBRIUM CONSTANT FOR PHOSPHORYLATION OF PHOSPHOGLUCOMUTASE AT 40°

Apparent equilibrium constants, $K_{app(t)}$, calculated from data obtained from incubations described in Table III.

Conditions assumed in calculations	EP_0 / *Enz*	K_{app} *Assuming Glc-1,6-$P_{2(t)}$ is universally labeled*			*Assuming Glc-1,6-$P_{2(t)}$ is 50% labeled during first 40 min*	
		10 min	*40 min*	*180 min*	*10 min*	*40 min*
Complete equilibration of ^{32}P on enzyme, using distribution data from Table III	0	65.3	68	32.9	32.7	34.0
	1	103	106	50.3	51.5	53.0
Incomplete equilibration of ^{32}P on enzyme, and $E_{(t)}$ = Glc-1,6-$P_{2(t)}$	1	1012	1179	305	235	275

$K_{app(40)}$ are much larger than $K_{app(180)}$. Moreover, the results show that the $K_{app(t)}$ values calculated for Condition b, incomplete equilibration of the phosphate of Glc-1,6-P_2 during the first 40 min, remain essentially constant throughout the time periods studied.

Regardless of which assumptions were made in evaluating the apparent equilibrium constant from the experimental data, its value was at least an order of magnitude greater than 3.76, the value previously reported[7]. In order to determine the source of the difference between the values obtained experimentally and the values predicted on the basis of the mechanism given by Eqns. 2 and 3 with $K = 3.76$, the equilibrium concentrations of $EP_{(eq)}$, $E_{(eq)}$, and Glc-1,6-$P_{2(eq)}$, were calculated assuming: (a) $K = 3.76$; (b) Glc-6-P = 0.945 μmole/3.04 ml (*i.e.*, Glc-6-$P_{(eq)}$ = Glc-6-P_0—Glc-1,6-$P_{2(eq)}$); (c) $EP_{(eq)} = EP_0$—Glc-1,6-$P_{2(eq)}$; and (d) $E_{(eq)}$ = Glc-1,6-$P_{2(eq)}$. Based on this calculation, the concentration of glucose-1,6-P_2 and of dephosphoenzyme present at equilibrium is 0.124 μmole per 3.04 ml and the fraction of the enzyme present as phosphoenzyme at equilibrium is 0.398. A comparison of these figures with the experimentally determined values reveals that the major discrepancy is manifested in the concentration of Glc-1,6-P_2 formed. Approx. 0.1 of the calculated quantity (based on $K = 3.76$) is observed experimentally. Since the radioactive material balance for the 10-, 40-, and 180-min incubations was 100, 97, and 98%, respectively, it does not appear likely that this difference resulted from material lost during the isolation procedure.

Experimentally, one of the crucial issues is the finding that the amount of dephosphoenzyme at time t, $E_{(t)}$, is not equal to, but is in fact considerably greater than the amount of glucose 1,6-diphosphate present at time t. Three possible sources of dephosphoenzyme are obvious: (a) the result of phosphatase-like action on phosphoenzyme forming $E + P_i$; (b) the result of reaction of EP with Glc-6-P or dissociation of an $E \cdot$Glc-1,6-P_2 complex resulting in the formation of E in a concentration equal to that of Glc-1,6-P_2; and (c) the fraction of enzyme initially present as E. Although careful analysis revealed that small quantities of P_i could be detected in the trichloroacetic acid supernatants, the quantity of P_i present was much too small to explain

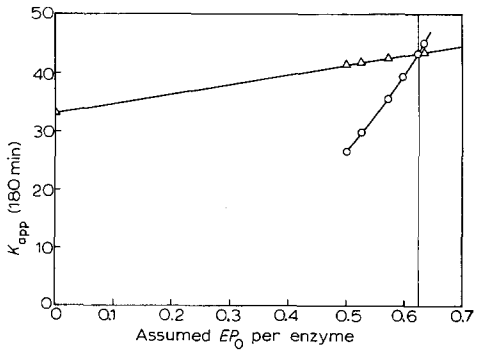


Fig. 3. Variation of apparent equilibrium constant with mole fraction of phosphoenzyme assumed to be present prior to incubation. $K_{app(180\ min)}$ defined as $\frac{(Glc\text{-}6\text{-}P)\ (EP)}{(Glc\text{-}1,6\text{-}P_2)\ (E)}$ following 180 min incubation. Reaction conditions described in legend of Table III. △ = $K_{app(180\ min)}$ calculated employing the radioactivity of the trichloroacetic acid-precipitated enzyme in determining the concentration of EP present after 180 min incubation; ○ = $K_{app(180\ min)}$ calculated based on EP = (assumed EP_0) − (Glc-1,6-P_2)$_{180}$.

the variation between the concentrations of Glc-1,6-$P_{2(t)}$ and $E_{(t)}$. It seems therefore that $E_{(t)}$ in excess of Glc-1,6-$P_{2(t)}$ must have resulted from dephosphoenzyme present prior to incubation, E_0. In order to estimate the concentration of E_0, a series of $K_{app(180)}$ values were calculated for several assumed fractions of phosphoenzyme prior to incubation, ranging from 0.50 to 0.635. For each assumed fraction of phosphoenzyme an isotopic dilution factor was calculated and used as the base for quantitating the μmoles of phosphate present in each reaction component after incubation. For each fraction of EP_0 assumed, two values for $K_{app(180)}$ were determined, one in which the quantitation of $EP_{(180)}$ and $E_{(180)}$ was based on the radioactivity of the trichloroacetic acid-precipitated protein, and a second in which the amount of dephosphoenzyme

TABLE V

FINAL REFINEMENT OF QUANTITATION OF SPECIES INVOLVED IN EQUILIBRIUM OF EQN. 3

Incubations described in legend of Table III. Refinement of quantitation used in calculating the values in this table was based on an initial mole fraction of phosphoenzyme, EP_0/enzyme, of 0.625, from which the final specific radioactivity of ^{32}P-labeled species was calculated.

	Quantitation based on					
	$EP_{(t)} = EP_0 - Glc\text{-}1,6\text{-}P_{2(t)}$ *and* $E_{(t)} = E_0 + Glc\text{-}1,6\text{-}P_{2(t)}$			*$EP_{(t)}$ and $E_{(t)}$ determined from radioactivity of trichloroacetic acid precipitates*		
Incubation time, t =	*10 min*	*40 min*	*180 min*	*10 min*	*40 min*	*180 min*
Glc-1,6-$P_{2(t)}$, μmoles	0.01278	0.01188	0.02241	0.01278	0.01188	0.02241
Glc-6-$P_{(t)}$, μmoles	0.9211	0.9253	0.9043	0.9211	0.9253	0.9043
$EP_{(t)}$, μmoles	0.1163	0.1172	0.1067	0.1128	0.1106	0.1065
$E_{(t)}$, μmoles	0.0903	0.0894	0.0999	0.0938	0.0960	0.1001
$K_{app(t)}$	92.9	102.2	43.1	86.7	89.7	42.9
$K_{app(t)}$ if Glc-1.6-P_2 is only 50% labeled during first 40 min	46.4	51.1	—	43.4	44.9	—

formed from phosphoenzyme during 180 min incubation was equal to the amount of Glc-1,6-P_2 formed (*i.e.*, $EP_{(180)} = EP_{0\,(assumed)} - \text{Glc-1,6-}P_{2\,(180)}$, and $E_{(180)} = E_{0\,(assumed)} + \text{Glc-1,6-}P_{2\,(180)}$). The results were plotted as shown in Fig. 3. The point of intersection, 0.625, represents the initial fraction of enzyme present as phosphoenzyme (EP_0/total enzyme). From this value all quantities were recalculated from the measured radioactivity of the isolated components, and the results are presented in Table V. The discrepancy between $K_{app\,(10)}$ and $K_{app\,(40)}$ compared with $K_{app\,(180)}$ persists if it is assumed that ^{32}P is completely randomized between positions 1 and 6 of Glc-1,6-P_2, and disappears if it is assumed that Glc-1,6-P_2 is only 50% labeled within 10 and 40 min of incubation.

As a consequence of these observations on the limited extent of dephosphorylation of the enzyme upon incubation with Glc-6-*P*, it became of interest to follow the transfer of [^{32}P]phosphate during the early phases of the phosphoglucomutase reaction. This was investigated by incubating ^{32}P-labeled phosphoenzyme with a large excess of unlabeled Glc-6-*P*. Particular care was exercised to remove any Glc-1-*P*, or other phosphate impurities that might be present in the Glc-6-*P* used in this experiment. The Glc-6-*P* was subjected to 1.0 M HCl in a boiling-water bath for 10 min prior to isolation by chromatography on Dowex 1-formate. The enzyme was incubated as indicated (Table VI), and each reaction was stopped by addition of trichloroacetic acid. The precipitated protein from each incubation mixture was prepared for counting, and the supernatant from each mixture, together with the first wash, was analyzed for Glc-1-*P*, Glc-6-*P*, Glc-1,6-P_2, and inorganic phosphate.

TABLE VI

REACTION OF ^{32}P-LABELED PHOSPHOGLUCOMUTASE WITH GLUCOSE 6-PHOSPHATE

Results indicate the distribution of ^{32}P-labeled phosphate among enzyme and glucose esters at end of each incubation, expressed as relative molar concentrations of each labeled species in per cent. Total ^{32}P-labeled enzyme incubated = 840 000–890 000 counts/min, $4.94 \cdot 10^{-4}$ M. Two ^{32}P-labeled glucose 1,6-diphosphate species are distinguished: glucose 1,6-[1-^{32}P]diphosphate labeled only in position 1 (radioactivity 100% acid labile) and glucose 1,6-[$^{32}P_2$]diphosphate labeled equally in positions 1 and 6 (50% acid labile).

Temp.	*Time (sec)*	*Concentration histidine and Mg^{2+} (M)*	*Per cent of total ^{32}P distributed as*				
			EP	*Glc-1-P*	*Glc-6-P*	*Glucose 1,6-[1-^{32}P]-diphosphate*	*Glucose 1,6-[$^{32}P_2$]-diphosphate*
0	4	0	94.5	4.85	0.00	0.68	0.000
0	10	0	87.8	11.52	0.00	0.67	0.005
0	4	0.0125	26.0	60.0	11.94	2.01	0.08
30	43	0.0125	17.44	5.68	71.0	0.42	5.43
30	3600	0.0125	11.11	5.65	81.1	0.07	2.09

The radioactive material balance of the individual chromatograms ranged from 99.0 to 99.9%. The results are presented in Table VI. The reaction at 0° in the absence of added histidine and Mg^{2+} (4-sec and 10-sec incubations) was considerably slower than the reaction at 0° in the presence of histidine and Mg^{2+}. These three incubations were performed in the presence of a large excess of Glc-6-*P*, the ratio of Glc-6-*P*:^{32}P-

labeled phosphoenzyme being 1000:1. At this ratio, upon complete equilibration of the radioactive phosphate a maximum of 0.1% of the ^{32}P would be associated with the protein. The first three experiments given in Table VI represent the ^{32}P distribution in the early phase of the reaction.

Considering that there is a large excess of Glc-6-*P*, if the two-step mechanism represented by Eqns. 2 and 3 were operating one would predict that in the early phase of the phosphoglucomutase reaction ^{32}P-labeled phosphate would accumulate in position 1 of Glc-1,6-P_2 before appearing as glucose 1-[^{32}P]phosphate. The results indicate that when 5.5% of the [^{32}P]phosphate had been transferred from the phosphoenzyme, the ratio of ^{32}P in Glc-1-*P* to that in position 1 of Glc-1,6-P_2 was 7.2, and after 10 sec when 12.7% had been transferred, the ratio was 17.2, suggesting that transfer of phosphate between phosphoenzyme and Glc-6-*P* to form Glc-1-*P* occurs without the obligatory formation of free Glc-1,6-P_2. Moreover, since the only pathway in this mechanism for the formation of glucose 6-[^{32}P]phosphate is by reaction of glucose 1-[^{32}P]phosphate with ^{32}P-labeled *EP* to form glucose 1,6-[^{32}P]diphosphate, one would predict the accumulation of ^{32}P in Glc-6-*P* to lag, so that in the early phase of the reaction the ratio of glucose 1-[^{32}P]phosphate to glucose 6-[^{32}P]phosphate would be greater than or at least equal to the ratio of glucose 1,6-[1-^{32}P]diphosphate to glucose 1,6-[$^{32}P_2$]diphosphate. An evaluation of the results indicates that where 74% of the ^{32}P has been transferred from the phosphoenzyme, the ratio of glucose 1-[^{32}P]phosphate to glucose 6-[^{32}P]phosphate was 5.02 while the ratio of glucose 1,6-[1-^{32}P]diphosphate to glucose 1,6-[$^{32}P_2$]diphosphate was 50. At this stage the ratio of glucose 1-[^{32}P]phosphate to glucose 1,6-[1-^{32}P]diphosphate was 30.5 and the ratio of glucose 6-[^{32}P]phosphate to glucose 1,6-[$^{32}P_2$]diphosphate was 136. Again the results suggest that transfer of phosphate in the phosphoglucomutase reaction can occur without the obligatory formation of free glucose 1,6-diphosphate.

Expts. 5 and 6 (Table VI) in this series were performed at 30° at a ratio of Glc-6-*P* to ^{32}P-labeled enzyme of 4.0. Under these conditions the phosphoglucomutase-catalyzed reaction is considerably faster, and although complete equilibration has not occurred within 60 min, 96% of the Glc-1,6-P_2 is labeled in both the 1 and 6 positions.

DISCUSSION

Various attempts to prepare completely dephosphorylated phosphoglucomutase in a single step by incubating the enzyme with a large excess of glucose 6-phosphate have been unsuccessful. Whether the procedure involved separation of the protein from the reaction mixture by the addition of trichloroacetic acid or separation by dialysis, similar results were obtained. In a few instances in early experiments dephosphorylation did occur where the enzyme was dialyzed overnight following incubation. This observation appeared to be associated with a phosphatase-like activity since the percentage of $^{32}P_i$ in the dialysate increased with time. A review of the data obtained in such experiments indicated that the less pure the enzyme preparation, the greater the phosphatase activity. The appearance of such phosphatase activity upon prolonged dialysis might explain the conflict between the data reported here and previous reports of extensive dephosphorylation[7,15].

The experimental findings presented in Table I reveal that a considerably greater fraction of phosphoenzyme remains after reaction than would be predicted

on the basis of the equilibrium reported for Reaction 3 (see ref. 7). Investigation of the apparent equilibrium constant as described above shows that this constant is at least an order of magnitude greater than that previously reported. When the experimentally observed concentrations of equilibrating species were compared with those predicted by an equilibrium constant of 3.76, it was found that the principal discrepancy was the final concentration of glucose 1,6-diphosphate. The amount detected experimentally was always less than that predicted. Moreover, the amount of dephosphoenzyme present after incubation was always greater than the amount of Glc-1,6-P_2. The conditions necessary to produce equal amounts of Glc-1,6-P_2 and dephosphoenzyme are: (a) that all the phosphoglucomutase be phosphoenzyme prior to incubation, and (b) that the enzyme preparation be free of phosphatase activity. An evaluation of the possibilities suggested by these requirements revealed that the phosphatase activity was minimal and was not sufficient to account for the amount of dephosphoenzyme in excess of the Glc-1,6-P_2, and the phosphoglucomutase consisted of approx. 1/3 dephosphoenzyme prior to incubation. If it were assumed that the original enzyme was entirely phosphoenzyme and that the quantity of Glc-1,6-P_2 produced during incubation was equal to the quantity of phosphoenzyme dephosphorylated, the apparent equilibrium constant would have been several orders of magnitude greater than that previously reported. In the previous reports[7] the assumption was made that the enzyme crystallized from muscle was all phosphorylated, the compound quantitated was Glc-1,6-P_2, and the quantity of dephosphoenzyme present at equilibrium was assumed to equal the Glc-1,6-P_2. Thus the site of conflict appears to be in the quantitation of Glc-1,6-P_2, since the previous investigators observed more of that species. The possibility of contamination of phosphoglucomutase with glucose-1-phosphate transphosphorylase, the enzyme which catalyzes the reaction

$$2\ \text{Glc-1-}P \rightleftharpoons \text{Glc-1,6-}P_2 + \text{glucose}$$

could explain this discrepancy. Both enzymes are present in rabbit skeletal muscle[12,17]. Although they are extracted by different solvent systems, some cross-contamination might be possible, and the presence of transphosphorylase activity in phosphoglucomutase preparations could provide a means for added Glc-1,6-P_2 accumulation. This possibility was investigated by incubating two enzyme preparations (one chromatographed at pH 6 and less pure, and the second chromatographed at pH 7) with ^{14}C-labeled Glc-6-P. Only a very small amount of [^{14}C]glucose, considerably less than the quantity of [^{14}C]glucose 1,6-diphosphate, could be detected after incubation with the less pure enzyme preparation, and no free [^{14}C]glucose was obtained after incubation with the pH 7-chromatographed phosphoglucomutase. The small quantity of [^{14}C]-glucose detected using the preparation chromatographed at pH 6 suggests that the transphosphorylase could be present in less pure preparations, and might account for the discrepancy between these results and those reported previously[7]. The finding that no [^{14}C]glucose could be detected when pH 7-chromatographed enzyme was incubated establishes a lack of transphosphorylase activity inherent in phosphoglucomutase *per se*.

Thus far the results have been discussed in terms of the two-step mechanism proposed by NAJJAR AND PULLMAN[3], represented by Eqns. 2 and 3. By following the transfer of radioactive phosphate from ^{32}P-labeled phosphoenzyme during the initial period of transfer, data were obtained which suggest that free Glc-1,6-P_2 is not an

obligatory intermediate in the phosphoglucomutase-catalyzed reaction. Such an interpretation is in agreement with that reported by RAY AND ROSCELLI[6].

In view of the results of their kinetic studies and our findings it is felt that the reversible transfer of phosphate from Glc-1-*P* to Glc-6-*P* can be mediated directly through phosphorylated phosphoglucomutase. A more extensive discussion of possible pathways for this transfer has been presented by these authors[6,18]. The results also suggest that the serylphosphate bond in the phosphoenzyme is more stable (of lower energy) than was previously indicated.

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