Biochem. J. (1981) **199**, 693–698
Printed in Great Britain

A stereochemical investigation of phosphoryl transfer catalysed by phosphoglucomutase by the use of α-D-glucose 1-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate

Gordon LOWE and Barry V. L. POTTER
Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.

(*Received 16 June 1981/Accepted 27 July 1981*)

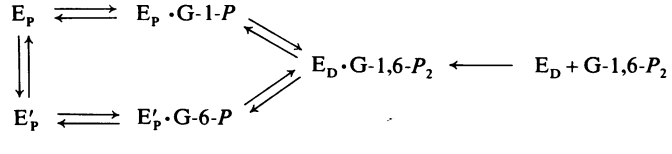
Rabbit muscle phosphoglucomutase converts α-D-glucose 1-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate into D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate, which is shown by ^{31}P n.m.r. spectroscopy, after cyclization and methylation, to have the (*S*)-configuration at the phosphorus atom. Since phosphoglucomutase is known to catalyse phosphoryl transfer by way of a phospho-enzyme intermediate, and since individual phosphoryl-transfer steps appear in general to occur with inversion of configuration, this observation is most simply interpreted in terms of a double-displacement mechanism with two phosphoryl-transfer steps.

Phosphoglucomutase catalyses the interconversion of α-D-glucose 1-phosphate and D-glucose 6-phosphate in the presence of a catalytic amount of D-glucose 1,6-bisphosphate (Caputto *et al.*, 1948; Leloir *et al.*, 1948; Cardini *et al.*, 1949), the equilibrium constant being 17 ± 2 in favour of D-glucose 6-phosphate at 25°C and pH 7.0 in the presence of 25 mM-Mg^{2+} (Atkinson *et al.*, 1961). The rabbit muscle enzyme exists in both a phospho and a dephospho form, the phospho-enzyme reacting with the D-glucose monophosphates but not with D-glucose 1,6-bisphosphate, whereas the dephospho-enzyme reacts with D-glucose 1,6-bisphosphate but not with the D-glucose monophosphates (Najjar & Pullman, 1954). The free dephospho-enzyme is not, however, formed during the catalytic cycle, since D-glucose 1,6-bisphosphate transfers a phosphoryl group to the enzyme more rapidly than it dissociates (Ray & Roscelli, 1964).

Strong support for this reaction pathway was provided by an investigation of the rates of isotope exchange at equilibrium, which showed that glucose is transferred just twice as fast as phosphate between substrate and products pools, only one catalytic cycle being required to transfer glucose, whereas two are required to transfer phosphate (Britton & Clark, 1968).

The existence of an active stable phospho-enzyme has allowed the phosphorylated amino acid to be identified as a serine residue and the sequence around it to be determined (Milstein & Sanger, 1961). Whether the phosphoryl group is transferred directly to both D-glucose 1-phosphate and D-glucose 6-phosphate is, however, an important question. Isotope transport experiments indicate that two phospho-enzyme intermediates are involved, E_P being formed by transfer of the 6-phosphate group and E'_P by transfer of the 1-phosphate group from D-glucose 1,6-bisphosphate. The reaction pathway can therefore be formulated as shown in Scheme 1. The apparent rate constant for isomerization of E'_P to E_P is about $4.5 \times 10^7 s^{-1}$, which is fast for a covalent change even by an enzyme, and was regarded as representing a conformational change of the phospho-enzyme (Britton & Clark, 1968).

If only a single covalent phospho-enzyme inter-



Scheme 1. *Proposed reaction pathway for phosphoglucomutase*
E_P and E'_P are phospho-enzyme intermediates, E_D is the dephospho-enzyme, and G-1-*P*, G-6-*P* and G-1,6-P_2 are α-D-glucose 1-phosphate, α-D-glucose 6-phosphate and α-D-glucose 1,6-bisphosphate respectively.

0306-3275/81/120693-06$01.50/1

mediate is involved and E_P and E'_P are conformational isomers, two phosphoryl-transfer steps would be required to convert D-glucose 1-phosphate into D-glucose 6-phosphate. If the reasonable assumption is made that the stereochemical courses of the two steps are the same, the interconversion should occur with retention of configuration at the phosphorus atom. If, however, an odd number of phosphoryl-transfer steps is involved, which would be so in the case of two covalently distinct phospho-enzyme intermediates, then inversion of configuration at the phosphorus atom would be expected. We have investigated the stereochemical course of phosphoryl transfer catalysed by phosphoglucomutase with the use of α-D-glucose 1-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate, in order to distinguish between these possibilities.

Materials and methods

Rabbit muscle phosphoglucomutase was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) as a crystalline suspension in 2.5M-$(NH_4)_2SO_4$, with an activity of 190units (μmol/min)/mg. D-Glucose 6-phosphate dehydrogenase, α-D-glucose 1,6-bisphosphate and 2,3,4,6-tetrabenzyl-α-D-glucose were also obtained from Sigma Chemical Co. (1*R*,2*S*)-1,2-[1-^{18}O]Dihydroxy-1,2-diphenylethane containing 94 atom % of ^{18}O at position 1 (we are grateful to Dr. P. M. Cullis for preparing this material) and $P^{17}OCl_3$ containing 3.3 atom % of ^{16}O, 43.5 atom % of ^{17}O and 53.2 atom % of ^{18}O were prepared as previously described (Cullis & Lowe, 1978, 1981).

Enzyme assays and u.v.-absorption measurements were performed on a Unicam SP.1800 spectrophotometer. ^{31}P n.m.r. spectra were recorded on a Bruker WH300FT spectrometer with quadrature detection at 121.5MHz, except for routine spectra, which were recorded on a Bruker WH90FT spectrometer at 36.43MHz. All spectra were proton-noise-decoupled except where otherwise indicated, and the chemical shifts (δ_P) were measured with reference to external trimethyl phosphate; ^2H_2O was used as a lock; signals downfield from the reference were assigned positive chemical shifts. pH measurements were made on a Radiometer PHM84 pH-meter.

trans-2-(2,3,4,6-Tetrabenzyl-1-D-glucosyl)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan

To a solution of freshly distilled $POCl_3$ (91μl, 1mol) in anhydrous pyridine (1ml) at 0°C was added dropwise, with stirring over 2h, a solution of *meso*-hydrobenzoin (214mg, 1mmol; thoroughly dried *in vacuo* over P_2O_5) in anhydrous pyridine (2ml). The mixture was allowed to reach room temperature over 1h, and 2,3,4,6-tetrabenzyl-D-glucose [541mg, 1mmol; thoroughly dried *in vacuo* over P_2O_5; $[\alpha]_{20}^{D}$ + 18.4°, literature value (Glaudemans & Fletcher, 1972) +21.7° for pure α-anomer; a 300MHz ^1H n.m.r. spectrum showed the α/β ratio to be 4:1] in anhydrous pyridine (2ml) was added dropwise to the stirred solution over a few minutes. After 4½h at room temperature (this optimum time was established by ^{31}P n.m.r. spectroscopy), the pyridine was evaporated and the final traces were removed by addition and evaporation of dioxan (2 × 10ml). N.m.r.: δ_P (pyridine, external ^2H_2O lock) +9.7 (triester of α-anomer, undecoupled q $^3J_{PH} = 7.5$Hz), +10.5 (triester of β-anomer, undecoupled q $^3J_{PH} = 7.5$Hz) and +12.5p.p.m. (cyclic five-membered diester, undecoupled t $^3J_{PH} = 7.0$Hz), the assignments being made in the knowledge that the α-anomer predominated.

D-*Glucose 1-phosphate*

trans-2-(2,3,4,6-Tetrabenzyl-1-D-glucosyl)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan was dissolved in anhydrous dioxan, and the solution (removed from the insoluble pyridinium hydrochloride by syringe) was added dropwise under dry N_2 to a rapidly stirred solution of Na (276mg, 12mmol) dissolved in anhydrous liquid NH_3 (50ml, dried by distillation from Na under dry N_2). After 15min NH_4Cl was added until the blue colour was discharged. The NH_3 was evaporated in a stream of dry N_2, and the residue was partitioned between ether and water. The aqueous layer was assayed for α-D-glucose 1-phosphate by the coupled enzyme assay with phosphoglucomutase and D-glucose 6-phosphate dehydrogenase (Bodansky, 1961; Ray & Roscelli, 1964). This assay showed the presence of 220μmol of α-D-glucose 1-phosphate (22% yield). The solution of D-glucose 1-phosphate (100ml) was adjusted to the pH and ionic strength of the triethylammonium bicarbonate buffer (25mM, pH7.6) used to equilibrate a column (100ml) of DEAE-Sephadex A-25. The solution was applied to the column and the column was developed with a linear gradient of triethylammonium bicarbonate (25–250mM) over 16h at a flow rate of 82ml/h. D-Glucose 1-phosphate (detected by the coupled enzyme assay) was eluted in fractions 45–56 (fractions collected every 15min), which were pooled and evaporated to dryness *in vacuo*, followed by addition and evaporation of methanol (50ml) to give bis-triethylammonium D-glucose 1-phosphate (210μmol, 21%). N.m.r.: δ_P (100mM-2-amino-2-methylpropane-1,3-diol hydrochloride, 10mM-EDTA, 25% ^2H_2O, pH9.0) +0.86 (α-D-glucose 1-phosphate) and +0.82 (β-D-glucose 1-phosphate) in the ratio 3:2, with the nuclear Overhauser effects being neglected; δ_H (^2H_2O) +3.0 to 3.8 (all protons except H_{C_1}), +4.8 (t, $^3J_{HH} = {}^3J_{PH} = 7.4$Hz, H_{C_1} of β-D-glucose 1-phos-

phate) and +5.3 p.p.m. (dd, $^3J_{HH} = 3.5$ Hz, $^3J_{PH} = 7.4$ Hz, H_{C_1} of α-D-glucose 1-phosphate).

D-Glucose 6-phosphate: phosphoglucomutase-catalysed phosphoryl transfer from α-D-glucose 1-phosphate

To a solution of bis-triethylammonium D-glucose 1-phosphate (200 μmol) in buffer (15 ml) (100 mM-Tris / 50 mM-imidazole / 5 mM-$MgCl_2$ / 1 mM-EDTA, pH 7.6) was added α-D-glucose 1,6-bisphosphate (1 mg, 1.24 μmol) and phosphoglucomutase (50 μl, 25 units). The solution was incubated at room temperature for 2.5 h, and then the enzyme was denatured by shaking the solution with chloroform. After extraction of the chloroform layer with buffer, the combined aqueous layers were adjusted to the pH and ionic strength of the triethylammonium bicarbonate buffer (25 mM, pH 7.6) used to equilibrate a column (100 ml) of DEAE-Sephadex A-25. The solution was applied to the column and the product was eluted with a linear gradient of triethylammonium bicarbonate buffer (25–200 mM) over 16 h at a rate of 82 ml/h. D-Glucose 6-phosphate [detected by enzyme assay with D-glucose 6-phosphate dehydrogenase (Lang & Michal, 1974)] was eluted in fractions 50–59 (fractions collected every 15 min), which were pooled and evaporated to dryness *in vacuo*, followed by addition and evaporation of methanol (2 × 50 ml) to give triethylammonium D-glucose 6-phosphate (134 μmol). N.m.r.: δ_P (^2H_2O) +2.5 (D-glucose 6-phosphate) and +0.5 p.p.m. (β-D-glucose 1-phosphate) in the ratio of 3:2, with the nuclear Overhauser effects being neglected. The mixture of D-glucose 6-phosphate and β-D-glucose 1-phosphate was converted into the mono-tri-*n*-octylammonium salts and then subjected to the cyclization and methylation procedure developed for the analysis of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate (Jarvest *et al.*, 1981). The ^{31}P n.m.r. spectrum showed that the products derived from β-D-glucose 1-phosphate would not interfere with the analysis.

(2R,4S,5R)-2-(2,3,4,6-Tetrabenzyl-1-D-glucosyl)-[2-^{17}O]oxo-4,5-diphenyl-[1-^{18}O]1,3,2-dioxaphospholan

This compound was prepared by the method described for *trans*-2-(2,3,4,6-tetrabenzyl-1-D-glucosyl)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan except that $P^{17}OCl_3$ was used in place of $POCl_3$ and (1*R*,2*S*)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane was used in place of *meso*-hydrobenzoin.

D-Glucose 1-[(S)-^{16}O,^{17}O,^{18}O]phosphate

This was prepared as described for D-glucose 1-phosphate except that (2*R*,4*S*,5*R*)-2-(2,3,4,6-tetrabenzyl-1-D-glucosyl)-2-[^{17}O]oxo-4,5-diphenyl-[1-^{18}O]1,3,2-dioxaphospholan replaced *trans*-2-(2,3,4,6-tetrabenzyl-1-D-glucosyl)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan.

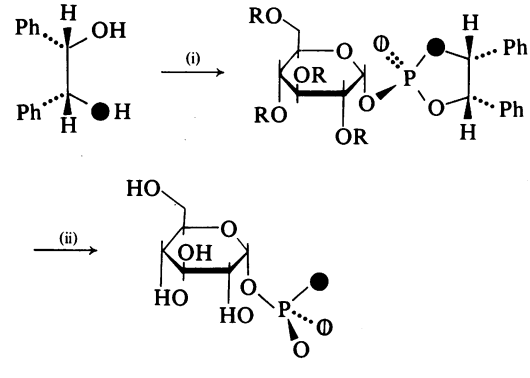
D-Glucose 6-[^{16}O,^{17}O,^{18}O]phosphate

This was prepared from D-glucose 1-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate by phosphoglucomutase-catalysed phosphoryl transfer in the presence of α-D-glucose 1,6-bisphosphate as described for D-glucose 6-phosphate.

Results and discussion

α-D-Glucose 1-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate was prepared by the general method of synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate esters (Cullis & Lowe, 1978, 1981) as outlined in Scheme 2. The 2,3,4,6-tetrabenzyl-D-glucose used in the systhesis was shown by ^1H n.m.r. spectroscopy to be a mixture of the α- and β-anomers in a ratio of approx. 4:1. In a preliminary experiment with the unlabelled phosphorylating agent, *trans*-2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan (prepared *in situ* from *meso*-hydrobenzoin and $POCl_3$ in pyridine), it was found that some anomerization took place, so that the D-glucose 1-phosphate obtained after reductive cleavage with Na in liquid NH_3 consisted of a mixture of α-D-glucose 1-phosphate and β-D-glucose 1-phosphate in the ratio of approx. 3:2. (Catalytic hydrogenolysis was very slow to reach completion, and gave the product in poor yield.)

Only the α-anomer of D-glucose 1-phosphate is a substrate for phosphoglucomutase (Cori *et al.*, 1937), so that, on incubation of the mixture of anomers of D-glucose 1-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate with phosphoglucomutase and a catalytic amount of



Scheme 2. *Synthesis of α-D-glucose 1-[(S)-^{16}O,^{17}O,^{18}O]-phosphate*

R = CH_2–Ph; ⓪ = ^{17}O, ● = ^{18}O. Reagents: (i) (*a*) $P⓪Cl_3$, C_5H_5N; (*b*) 2,3,4,6-tetrabenzyl-D-glucose, C_5H_5N; (ii) Na, liquid NH_3.

α-D-glucose 1,6-bisphosphate, the α-anomer is converted (almost completely) into D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate, whereas the β-anomer is unchanged.

We have developed a ^{31}P n.m.r. method for analysing the chirality at the phosphorus atom of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate after cyclization followed by methylation to the isotopically labelled axial and equatorial methyl D-glucose 4,6-bisphosphate triesters (Jarvest *et al.*, 1981). In a preliminary experiment with unlabelled materials we showed that the products derived from β-D-glucose 1-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate that would be present with those derived from D-glucose 6-[^{16}O,^{17}O,^{18}O]-phosphate would not interfere with the ^{31}P n.m,r. analysis, since they would resonate at a different chemical shift.

The ^{31}P n.m.r. spectrum of the axial and equatorial triesters derived by cyclization and methylation of the D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate obtained by the phosphoglucomutase-catalysed isomerization of α-D-glucose 1-[^{16}O,^{17}O,^{18}O]phosphate is shown in Fig. 1; the assignments are shown on the spectrum. From the ratio of the [$^{16}O_{ax.}$,$^{18}O_{eq.}$]- to [$^{16}O_{eq.}$,$^{18}O_{ax.}$]-axial and -equatorial triesters it follows (Jarvest *et al.*, 1981) that D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate derived from α-D-glucose 1-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate by the phosphoglucomutase-catalysed reaction has the (*S*)-configuration at the phosphorus atom and hence the phosphoryl transfer has occurred with retention of configuration. Since the isotopic compositions of the (1*R*,2*S*)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane and $P^{17}OCl_3$ are known, and the amount of α-D-glucose 1,6-bisphosphate (approx. 2%) used as co-substrate (which will dilute the isotopes) is also known, it is possible to calculate [assuming 4% loss of isotope during the cyclization step (Jarvest *et al.*, 1981)] the expected relative intensities of the isotopically labelled triesters in the ^{31}P n.m.r. spectrum for phosphoryl transfer with retention and inversion of configuration at the phosphorus atom. These are compared in Table 1 with the observed relative intensities, from which we can say that the phosphoryl transfer catalysed by phosphoglucomutase has occurred with retention of configuration at the phosphorus atom with a stereoselectivity in excess of 94%.

In view of the established intermediacy of a phospho-enzyme on the reaction pathway of the phosphoglucomutase-catalysed reaction, a minimum of two phosphoryl-transfer steps must be involved. Although the observed retention of configuration at the phosphorus atom could be inter-

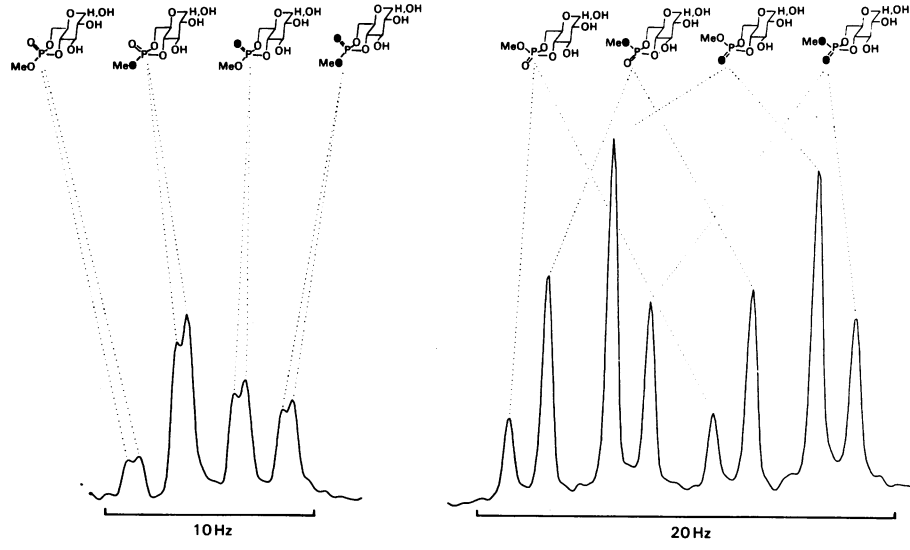


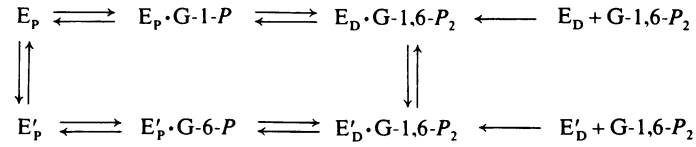
Fig. 1. *^{31}P n.m.r. spectrum (121.5 MHz, in 33% dimethyl sulphoxide and 66% [$^2H_4$]methanol containing 8-hydroxyquinoline) of the equatoral and axial triesters derived by cyclization followed by methylation of* D-*glucose 6-[^{16}O,^{17}O,^{18}O]phosphate obtained by the phosphoglucomutase-catalysed phosphoryl transfer from* D-*glucose 1-[(S)-^{16}O,^{17}O,^{18}O]phosphate*

The ^{31}P n.m.r. parameters are: offset 2240 Hz, sweep width 2000 Hz, pulse width (angle) 16 μs (75°), acquisition time 2.05 s, memory size 8 K, broad-band proton-noise decoupling, gaussian multiplication (line broadening −0.9 Hz, gaussian broadening 0.4) in 8 K and Fourier transform in 32 K. ● = ^{18}O.

Table 1. *Observed relative peak intensities of the ^{31}P n.m.r. resonances (from Fig. 1, average of both anomers) of the ^{18}O-labelled diastereoisomeric triesters derived by cyclization followed by methylation of the D-glucose 6-[^{16}O,^{17}O,^{18}O]-phosphate, and the calculated values expected for the phosphoglucomutase-catalysed phosphoryl transfer with retention and inversion of configuration*

For full details see the text. ● = ^{18}O.

	Equatorial triester			Axial triester		
		Calculated			Calculated	
	Observed	Retention	Inversion	Observed	Retention	Inversion
MeO–P=O	0.23	0.24	0.24	0.25	0.24	0.24
Me●–P=O	1.00	1.00	0.65	0.63	0.65	1.00
MeO–P=●	0.66	0.65	1.00	1.00	1.00	0.65
Me●–P=●	0.54	0.61	0.61	0.56	0.51	0.51



Scheme 3. *Modified reaction pathway for phosphoglucomutase in which E_P and E'_P are conformational isomers of the phospho-enzyme and E_D and E'_D are conformational isomers of the dephospho-enzyme*

Other abbreviations are the same as in Scheme 1.

preted in terms of either two steps occurring with retention of configuration or two steps occurring with inversion of configuration, in view of the growing evidence that phosphoryl transfer catalysed by phosphokinases that adopt a sequential pathway occurs with inversion of configuration (Knowles, 1980; Lowe *et al.*, 1981) it seems that single phosphoryl-transfer steps are catalysed with inversion of configuration. It seems reasonable therefore to suggest that there are two [higher even numbers can be excluded from isotope transport experiments (Britton & Clark, 1968)] phosphoryl-transfer steps in the phosphoglucomutase reaction, and that they occur with inversion of configuration, leading to overall retention of configuration at the phosphorus atom. It follows, therefore, that the two phospho-enzyme intermediates E_P and E'_P in Scheme 1 are conformational isomers with the phosphoryl group covalently linked to the same active-site serine hydroxy group, as was suggested by isotope transport studies (Britton & Clark, 1968). It is noteworthy that phosphoroglycerate mutase also catalyses phosphoryl transfer with retention of configuration at the phosphorus atom, for both the 2,3-bisphospho-D-glycerate-dependent rabbit muscle enzyme and the cofactor-independent wheat-germ enzyme (Blättler & Knowles, 1980).

Evidence has also been found for the existence of the E_D–glucose 1,6-bisphosphate complex in two states (Ray *et al.*, 1973; Ray & Mildvan, 1973; Ma & Ray, 1980), and it was suggested that these may involve a re-orientation of α-D-glucose 1,6-bisphosphate on the enzyme without dissociation from it (the exchange mechanism). In view of the established conformational isomerization of the phospho-enzyme, it seems structurally more attractive to suggest that a conformational isomerization of the dephospho-enzyme can occur, in the presence or in the absence of α-D-glucose 1,6-bisphosphate, so that the stereochemically demanding 'in-line' phosphoryl transfer to the active-site serine residue can be achieved from the 1-position in one conformation (E_D) and the 6-position from the other conformation (E'_D). The reaction pathway would then be represented as shown in Scheme 3.

Such a reaction pathway makes good sense, since both substrates (and co-substrate) have the same carbon–oxygen skeleton, so that by a suitable conformational change it is only necessary to evolve one binding site. It may also be relevant that the phosphoryl-transfer potential of the substrates, especially D-glucose 6-phosphate, is low, so that the intervention of a covalent phospho-enzyme intermediate could have a catalytic advantage. So, by providing a refuge for the phosphoryl group on the enzyme, the overall activation energy for phosphoryl transfer should be lowered, and the need to evolve more than one substrate-binding site unnecessary.

We gratefully acknowledge financial support from the Science Research Council. This paper is a contribution from the Oxford Enzyme Group, supported by the Science Research Council.

References

Atkinson, M. R., Johnson, E. & Morton, R. K. (1961) *Biochem. J.* **79**, 12–15

Blättler, W. A. & Knowles, J. R. (1980) *Biochemistry* **19**, 738–743

Bodansky, O. (1961) *J. Biol. Chem.* **236**, 328–332

Britton, H. G. & Clark, J. B. (1968) *Biochem. J.* **110**, 161–179

Caputto, R., Leloir, L. F., Trucco, R. E., Cardini, C. E. & Paladini, A. (1948) *Arch. Biochem.* **18**, 201–203

Cardini, C. E., Paladini, A. C., Caputto, R., Leloir, L. F. & Trucco, R. E. (1949) *Arch. Biochem.* **22**, 87–100

Cori, C. F., Colowick, S. P. & Cori, G. T. (1937) *J. Biol. Chem.* **121**, 465–477

Cullis, P. M. & Lowe, G. (1978) *J. Chem. Soc. Chem. Commun.* 512–514

Cullis, P. M. & Lowe, G. (1981) *J. Chem. Soc. Perkin Trans. 1* 2317–2321

Glaudemans, C. P. J. & Fletcher, H. G., Jr. (1972) *Methods Carbohydr. Chem.* **6**, 373–376

Jarvest, R. L., Lowe, G. & Potter, B. V. L. (1981) *J. Chem. Soc. Perkin Trans. 1* in the press

Knowles, J. R. (1980) *Annu. Rev. Biochem.* **49**, 877–919

Lang, G. & Michal, G. (1974) in *Methods of Enzymatic Analysis*, 2nd English edn. (Bergmeyer, H. U., ed.), vol. 3, pp. 1238–1242, Academic Press, New York and London

Leloir, L. F., Trucco, R. E., Cardini, C. E., Paladini, A. C. & Caputto, R. (1948) *Arch. Biochem.* **19**, 339–340

Lowe, G., Cullis, P. M., Jarvest, R. L., Potter, B. V. L. & Sproat, B. S. (1981) *Philos. Trans. R. Soc. London Ser. B* **293**, 75–92

Ma, C. & Ray, W. J., Jr. (1980) *Biochemistry* **19**, 751–759

Milstein, C. & Sanger, F. (1961) *Biochem. J.* **79**, 456–469

Najjar, V. A. & Pullman, M. E. (1954) *Science* **119**, 631–634

Ray, W. J., Jr. & Mildvan, A. S. (1973) *Biochemistry* **12**, 3733–3743

Ray, W. J., Jr. & Roscelli, G. A. (1964) *J. Biol. Chem.* **239**, 1228–1236

Ray, W. J., Jr., Mildvan, A. S. & Long, J. W. (1973) *Biochemistry* **12**, 3724–3732