The stereochemical course of phosphoryl transfer catalysed by glucose 6-phosphatase

Gordon LOWE and Barry V. L. POTTER

Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.

(Received 9 November 1981/Accepted 14 December 1981)

Rat liver microsomal glucose 6-phosphatase catalyses phosphoryl transfer between D-glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate and D-glucose with retention of configuration at the phosphorus atom. 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 a phosphoryl-enzyme intermediate. Such an intermediate has been proposed previously from kinetic and ^{32}P -labelling experiments.

Glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) occupies a strategic position in carbohydrate metabolism, since glucose 6-phosphate is an intermediate in several metabolic pathways. The location of the enzyme in the membrane of the endoplasmic reticulum of liver, kidney and intestine suggests that the enzyme is involved in glucose transport (Nordlie, 1971).

Although formally considered to be a hydrolytic enzyme, glucose 6-phosphatase possesses potent transferase activity, and under certain conditions this can exceed the rate of hydrolysis. The ability of liver microsomal glucose 6-phosphatase to catalyse phosphoryl transfer between glucose 6-phosphate and [^{14}C]glucose led to the suggestion that the enzyme catalyses both hydrolysis and phosphoryl transfer of glucose 6-phosphate by way of a phosphoryl-enzyme intermediate (Segal, 1959; Hass & Byrne, 1960*a,b*). Chemical evidence for a phosphoryl-enzyme intermediate was provided by incubating rat liver microsomal fraction with glucose 6-[(^{32}P)]phosphate, and demonstrating that ^{32}P was incorporated into protein as *N*-3-phosphorylhistidine (Feldman & Butler, 1969, 1972). However, it was not possible to establish that the phosphorylated protein was glucose 6-phosphatase or that the phosphoryl-enzyme was a kinetically competent intermediate. The evidence for a phosphoryl-enzyme intermediate is therefore much less secure than, for example, in the case of alkaline phosphatase from *Escherichia coli* (Reid & Wilson, 1971).

There is growing evidence to support the view that enzymes catalyse single phosphoryl-transfer steps with inversion of configuration at the phosphorus atom (Knowles, 1980; Lowe *et al.*, 1981). If therefore a phosphoryl-enzyme intermediate is in-

involved in glucose 6-phosphatase-catalysed hydrolysis and phosphoryltransferase reactions, two phosphoryl-transfer steps would be involved, which should lead to overall retention of configuration at the phosphorus atom. If, however, the hydrolytic activity involves a single phosphoryl-transfer step with inversion of configuration, then equilibration of D-glucose with D-glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate should lead to racemization of the chiral [^{16}O , ^{17}O , ^{18}O]phosphate group. A stereochemical investigation of the phosphoryltransferase activity of glucose 6-phosphatase was therefore undertaken in order to distinguish between these possibilities.

Materials and methods

Rat liver microsomal glucose 6-phosphatase was obtained from Dr. R. Keller (Sigma Chemical Co., St. Louis, MO, U.S.A.). One unit of enzyme produces 1 μmol of inorganic phosphate/min from glucose 6-phosphate at 37°C and pH 6.5. D-[U- ^{14}C]Glucose (50 μCi , 317 mCi/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Scintillation counting of radioactivity was performed in Luma Gel scintillant (5 ml samples) obtained from LKB Produkter and on an LKB 1215 Rackbeta liquid-scintillation counter. (1*R*,2*S*)-1,2-[1- ^{18}O]Dihydroxy-1,2-diphenylethane was chirally pure and contained 93 atom % ^{18}O (we are grateful to Dr. P. M. Cullis for this material) and $\text{P}^{17}\text{OCl}_3$ containing 3.3 atom % ^{16}O , 43.5 atom % ^{17}O and 53.2 atom % ^{18}O were prepared as previously described (Cullis & Lowe, 1981) and used to synthesize [γ (*S*)- ^{16}O , ^{17}O , ^{18}O]ATP as before (Lowe & Potter, 1981). The isotopomeric composition was established by ^{31}P n.m.r. spectroscopy to be

$[\gamma\text{-}^{16}\text{O}_3]\text{ATP}$ 15.1%, $[\gamma\text{-}^{16}\text{O}_2, ^{18}\text{O}]\text{ATP}$ 18.1%, $[\gamma\text{-}^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\text{ATP}$ 28.2% and $[\gamma\text{-}^{16}\text{O}, ^{18}\text{O}_2]\text{ATP}$ 38.6%.

D-Glucose 6-phosphate was assayed with D-glucose 6-phosphate dehydrogenase and NADP^+ (Lang & Michal, 1974). 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 WH 300 FT spectrometer with quadrature detection at 121.5 MHz, except for routine spectra, which were recorded on a Bruker WH 90 FT spectrometer at 36.43 MHz. All spectra were proton-noise-decoupled, and chemical shifts (δ) were measured with reference to external trimethyl phosphate; $^2\text{H}_2\text{O}$ was used as a lock; signals downfield from the reference were assigned positive chemical shifts. pH measurements were made on a Radiometer PHM 84 pH-meter.

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

D-Glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate was prepared by incubating $[\gamma(\text{S})\text{-}^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\text{ATP}$ and D-glucose with yeast hexokinase as described previously (Lowe & Potter, 1981); the reaction proceeds with inversion of configuration at the phosphorus atom.

Incubation of D-[U- ^{14}C]glucose and D-glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate with glucose 6-phosphatase

D-Glucose (3.6 mmol, 400 mM), D-[U- ^{14}C]glucose (47.4 μmol , 15 μCi) and D-glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate (0.22 mmol) were dissolved in 50 mM-triethanolamine hydrochloride buffer, pH 6.5 (9 ml). A portion (25 μl) of this solution in water (75 μl) was added to Luma Gel (5 ml), and the solution was found to have 5091 c.p.m./ μmol (counting efficiency approx. 56%). Glucose 6-phosphatase (3 units) was added, and the solution was incubated at 37°C. The D-glucose 6-phosphate hydrolysis was monitored by ^{31}P n.m.r. spectroscopy and by the glucose 6-phosphate dehydrogenase assay. After 3.5 h (the equilibration time had been established in a preliminary experiment) the mixture was acidified to pH 2 by the dropwise addition of 1 M-HCl and vigorously agitated with chloroform. After separation and filtration, the aqueous layer was adjusted to pH 8.0 with NaOH solution and applied to a column (100 ml) of DEAE-Sephadex A-25 that had been equilibrated with 25 mM-triethylammonium bicarbonate buffer, pH 8.0. The column was run with a gradient of 25–250 mM-triethylammonium bicarbonate buffer, pH 8.0, over 16 h at a flow rate of 82 ml/h. D-Glucose 6-[(^{16}O , ^{17}O , ^{18}O)]phosphate was eluted, and the fractions were pooled and evaporated; enzymic

assay showed there to be 0.105 mmol, which had a specific radioactivity of 5500 c.p.m./ μmol .

Analysis of the chirality at phosphorus of D-[U- ^{14}C]glucose 6-[(^{16}O , ^{17}O , ^{18}O)]phosphate

This was performed as previously described for D-glucose 6-[(S)- ^{16}O , ^{17}O , ^{18}O]phosphate (Jarvest *et al.*, 1981).

Results and discussion

D-Glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate was prepared enzymically by incubating $[\gamma(\text{S})\text{-}^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\text{ATP}$ and D-glucose with yeast hexokinase; the reaction is known to occur with inversion of configuration at the phosphorus atom (Lowe & Potter, 1981). D-Glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate was incubated with rat liver microsomal glucose 6-phosphatase in the presence of a high concentration of D-[U- ^{14}C]glucose (0.4 M) until equilibration of the radioactivity between D-glucose and D-glucose 6-[(^{16}O , ^{17}O , ^{18}O)]phosphate was complete. After this time approximately half of the D-glucose 6-[(^{16}O , ^{17}O , ^{18}O)]phosphate had been hydrolysed to D-glucose and [^{16}O , ^{17}O , ^{18}O]phosphate. If phosphoryl transfer between D-glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate and D-[U- ^{14}C]glucose occurs with inversion of configuration, by the time equilibration of the radioactivity has been achieved racemization of the chiral phosphoryl group should have occurred, since there is an equal probability that the product will be derived from an odd number as an even number of phosphoryl-transfer steps. If, however, phosphoryl transfer occurs by way of a phosphoryl-enzyme intermediate, the product can only be derived by an even number of phosphoryl-transfer steps, and hence the recovered D-glucose 6-[(^{16}O , ^{17}O , ^{18}O)]phosphate should retain the (R)-configuration at the phosphorus atom.

In order to determine the chirality at the phosphorus atom the recovered D-[U- ^{14}C]glucose 6-[(^{16}O , ^{17}O , ^{18}O)]phosphate was cyclized and methylated as previously described (Jarvest *et al.*, 1981); the ^{31}P n.m.r. spectrum is shown in Fig. 1. Since the axial and equatorial triesters derived from D-glucose 4,6-[($^{18}\text{O}_{\text{ax}}$, $^{16}\text{O}_{\text{eq}}$)]phosphate predominate over those derived from D-glucose 4,6-[($^{16}\text{O}_{\text{ax}}$, $^{18}\text{O}_{\text{eq}}$)]phosphate, phosphoryl transfer must have occurred with overall retention of configuration. The observed relative intensities from Fig. 1 are compared with the calculated values for retention of configuration in Table 1, and show no loss of chirality (within experimental error). If racemization had occurred, the intensity of the axial and equatorial triester resonances derived from D-glucose 4,6-[($^{18}\text{O}_{\text{ax}}$, $^{16}\text{O}_{\text{eq}}$)]phosphate and D-glucose 4,6-[($^{16}\text{O}_{\text{ax}}$, $^{18}\text{O}_{\text{eq}}$)]phosphate would have been the same.

The transfer of the [^{16}O , ^{17}O , ^{18}O]phosphoryl group

Fig. 1. ^{31}P n.m.r. spectrum (121.5 MHz) of the equatorial and axial triesters derived by cyclization followed by methylation of D-[U- ^{14}C]glucose 6-[^{16}O , ^{17}O , ^{18}O]phosphate obtained by incubating D-glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate with D-[U- ^{14}C]glucose and rat liver microsomal glucose 6-phosphatase

The solvent is 33% (v/v) dimethyl sulphoxide in [$^2\text{H}_4$]methanol. The ^{31}P n.m.r. parameters are: offset 2240 Hz, sweep width 2000 Hz, acquisition time 2.05 s, pulse width (angle) $16\mu\text{s}$ (75°), gaussian multiplication (line broadening -0.9 Hz , gaussian broadening 0.4) in 8 K and Fourier transform in 32 K. $\bullet = ^{18}\text{O}$.

Table 1. Comparison of the observed relative peak intensities for the ^{31}P resonances in Fig. 1 with the calculated values expected for the glucose 6-phosphatase-catalysed phosphoryl transfer with retention of configuration

The isotopically labelled diastereoisomeric triesters are derived by cyclization followed by methylation of the D-[U- ^{14}C]glucose 6-[^{16}O , ^{17}O , ^{18}O]phosphate and its isotopomers. The calculated values are those expected from the known isotopomeric composition of the ATP used to synthesize the D-glucose 6-[(R)- ^{16}O , ^{17}O , ^{18}O]phosphate. $\bullet = ^{18}\text{O}$.

	Equatorial triester		Axial triester	
	Observed	Calculated (retention)	Observed	Calculated (retention)
$\text{MeO}-\text{P}=\text{O}$	0.75	0.74	0.74	0.74
$\text{Me}\bullet-\text{P}=\text{O}$	0.63	0.67	1.00	1.00
$\text{MeO}-\text{P}=\bullet$	1.00	1.00	0.68	0.67
$\text{Me}\bullet-\text{P}=\bullet$	0.47	0.46	0.58	0.46

with overall retention of configuration by glucose 6-phosphatase provides strong evidence in support of the double-displacement mechanism involving a phosphoryl-enzyme intermediate. Alkaline phosphatase from *E. coli* (Jones *et al.*, 1978) and acid phosphatase from bovine liver (Saini *et al.*, 1981) also catalyse phosphoryl transfer with retention of configuration.

Although glucose 6-phosphatase shows greater specificity than alkaline or acid phosphatase, it is a

multifunctional enzyme and will hydrolyse a wide variety of phosphate esters and amides (Nordlie, 1971). Non-specific phosphatases presumably do not have a binding site for the leaving group, and, since the binding energy for water at the active site must be relatively small, it could be that it is necessary to have a covalent intermediate on the reaction pathway in order to lower the entropy of activation. This explanation is consistent with the finding that the specific enzyme cyclic AMP phos-

phodiesterase from both bovine heart and yeast catalyses the hydrolysis of cyclic AMP with inversion of configuration (Cullis *et al.*, 1981; R. L. Jarvest, G. Lowe, J. Baraniak & W. J. Stec, unpublished work), whereas the non-specific snake-venom phosphodiesterase catalyses the hydrolysis of phosphate diesters with retention of configuration (Jarvest & Lowe, 1981). However, it would be unwise to regard specificity as an infallible guide to mechanism, as is well illustrated by the ATPases: myosin and mitochondrial ATPases catalyse the hydrolysis of ATP with inversion of configuration at the phosphorus atom (Webb & Trentham, 1980; Webb *et al.*, 1980), whereas the sarcoplasmic-reticulum ATPase catalyses the hydrolysis of ATP with retention of configuration at the phosphorus atom (Webb & Trentham, 1981).

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

References

- Cullis, P. M., Jarvest, R. L., Lowe, G. & Potter, B. V. L. (1981) *J. Chem. Soc. Chem. Commun.* 245–246
- Feldman, F. & Butler, L. G. (1969) *Biochem. Biophys. Res. Commun.* **36**, 119–125
- Feldman, F. & Butler, L. G. (1972) *Biochim. Biophys. Acta* **268**, 698–710
- Hass, L. F. & Byrne, W. L. (1960a) *Science* **131**, 991–992
- Hass, L. F. & Byrne, W. L. (1960b) *J. Am. Chem. Soc.* **82**, 947–954
- Jarvest, R. L. & Lowe, G. (1981) *Biochem. J.* **199**, 447–451
- Jarvest, R. L., Lowe, G. & Potter, B. V. L. (1981) *J. Chem. Soc. Perkin Trans. I* 3186–3195
- Jones, S. R., Kindman, L. A. & Knowles, J. R. (1978) *Nature (London)* **275**, 564–565
- 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
- Lowe, G. & Potter, B. V. L. (1981) *Biochem. J.* **199**, 227–233
- 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
- Nordlie, R. C. (1971) *Enzymes 3rd Ed.* **4**, 543–610
- Reid, T. W. & Wilson, I. B. (1971) *Enzymes 3rd Ed.* **4**, 373–415
- Saini, M. S., Buchwald, S. L., Van Etten, R. L. & Knowles, J. R. (1981) *Biochemistry* **256**, 10453–10455
- Segal, H. L. (1959) *J. Am. Chem. Soc.* **81**, 4047–4050
- Webb, M. R. & Trentham, D. R. (1980) *J. Biol. Chem.* **255**, 8629–8632
- Webb, M. R. & Trentham, D. R. (1981) *J. Biol. Chem.* **256**, 4884–4887
- Webb, M. R., Grubmeyer, C., Penefsky, H. S. & Trentham, D. R. (1980) *J. Biol. Chem.* **255**, 11637–11639