

## The stereochemical course of phosphoryl transfer catalysed by glucokinase

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Adenosine 5'-[ $\gamma$ (S)- $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]triphosphate has been used to determine the stereochemical course of phosphoryl transfer catalysed by rat liver glucokinase. The chirality of the product, D-glucose 6-[ $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]phosphate was analysed by  $^{31}\text{P}$  n.m.r. spectroscopy. The reaction proceeds with inversion of configuration at phosphorus. The simplest interpretation of this result, which is the same as that observed with yeast hexokinase [Lowe & Potter (1981) *Biochem. J.* **199**, 227–233], is that the phosphoryl group is transferred between  $\text{MgATP}^{2-}$  and glucose in the ternary complex by an 'in-line' mechanism. It accords with the view that the kinetic differences between glucokinase and the other hexokinases arise from differences in rate constants and not from any fundamental differences in chemical mechanism.

Rat liver glucokinase (ATP:D-glucose 6-phosphotransferase; EC 2.7.1.2) differs substantially from other hexokinases (ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1) both in structure and in kinetic behaviour. Its molecular mass of 48 kDa (Holroyde *et al.*, 1976) is about half the values of 96–98 kDa reported for the other rat isoenzymes (Holroyde & Trayer, 1976; Wright *et al.*, 1978), and unlike them it shows positive co-operativity with respect to glucose (Niemeyer *et al.*, 1975; Storer & Cornish-Bowden, 1976) and is insensitive to inhibition by glucose 6-phosphate (Storer & Cornish-Bowden, 1977). Its properties have been reviewed more fully elsewhere (Pollard-Knight & Cornish-Bowden, 1982).

The main kinetic interest in glucokinase lies in its co-operativity, an unusual property in a monomeric enzyme (Holroyde *et al.*, 1976; Cárdenas *et al.*, 1978) with only a single active site (Connolly & Trayer, 1979). Efforts to explain this co-operativity in terms of a mnemonic mechanism (Storer & Cornish-Bowden, 1977) have received support from isotope-exchange experiments (Gregoriou *et al.*, 1981a): steady-state experiments indicated a compulsory order of substrate addition and product release, with glucose bound first and glucose 6-phosphate released last, as required by the mnemonic mechanism; equilibrium isotope-exchange experiments confirmed that interconversion of ternary complexes could not be rate-limiting, but suggested that the complete mechanism might be

somewhat more complex than a simple mnemonic mechanism, with some degree of randomness in the order of addition of substrates, as proposed by Cárdenas *et al.* (1979).

None of the proposals to account for the unusual kinetic behaviour of glucokinase require any fundamental difference in chemistry from the reactions catalysed by the other hexokinases, and indeed it is likely that the structural differences may be more apparent than real. The close similarity in amino acid composition between glucokinase and the muscle isoenzyme hexokinase type II (Holroyde & Trayer, 1976) suggests a degree of sequence similarity of the order of 85% (Cornish-Bowden, 1977). Thus, although hexokinase type II is also a monomeric enzyme, it may resemble a dimer of glucokinase, in the sense that its sequence may comprise two similar halves, of which one has retained a catalytic site, whereas the catalytic site of the other has evolved into an allosteric site (Trayer, 1981). If this is correct one would expect the chemical mechanism of glucokinase to be essentially the same as that of the other hexokinases. Partial confirmation has come from the observation that glucokinase is specific for the  $\beta\gamma$ -bidentate complex of  $\text{MgATP}^{2-}$  with the *A* screw sense (Darby & Trayer, 1981), in common with yeast hexokinase (Cornelius & Cleland, 1978). Moreover, flux-ratio studies of hexokinase type II show the same order of addition of substrates and release of products as that found to predominate for glucokinase (Gregoriou *et al.*, 1981b). The interconversion of ternary complexes appears to be rate-limiting for hexokinase type II.

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however, whereas it is not for glucokinase. In support of the idea that apparently qualitative differences in kinetic behaviour can be the result of quantitative differences in the rate constants of a common mechanism.

We now report further evidence of a unity of chemical mechanism between glucokinase and the other hexokinases, in the form of a determination of the stereochemical course of phosphoryl transfer catalysed by glucokinase. Information of this kind has not previously been available for any mammalian hexokinase, but yeast hexokinase provides a basis for comparison: it has been shown to catalyse phosphoryl transfer with inversion of configuration at phosphorus (Lowe & Potter, 1981).

## Materials and methods

### Glucokinase

The enzyme was purified to a specific activity of 17  $\mu$ kat/mg of protein by the methods described previously (Holroyde *et al.*, 1976). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed glucokinase as the major band; although one minor band was also present no contamination by other hexokinases was detectable. The enzyme was stored until use in 20 mM-Tris buffer at pH 7.5 containing 30% glycerol, 1 mM-EDTA, 1 mM-dithiothreitol, 50 mM-glucose and 0.4 M-KCl.

### Adenosine 5'-[ $\gamma$ (S)- $^{16}\text{O}$ , $^{17}\text{O}$ , $^{18}\text{O}$ ]triphosphate

This was prepared as described previously (Lowe & Potter, 1981) from  $\text{P}^{17}\text{OCl}_3$ , which was shown, by mass spectrometry after conversion of a portion with methanol into isotopically labelled trimethyl phosphate, to have an isotope composition of 3.3 atom%  $^{16}\text{O}$ , 43.5 atom%  $^{17}\text{O}$  and 53.2 atom%  $^{18}\text{O}$ . (1*R*,2*S*)-1,2-[1- $^{18}\text{O}$ ]Dihydroxy-1,2-diphenylethane used in the synthesis was chirally pure and contained 93 atom%  $^{18}\text{O}$ . From the  $^{31}\text{P}$  n.m.r. spectrum the isotopomeric composition was shown to be: [ $\gamma$ - $^{16}\text{O}_3$ ]ATP, 15.1%; [ $\gamma$ - $^{16}\text{O}_2$ ,  $^{18}\text{O}$ ]ATP, 18.1%; [ $\gamma$ - $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]ATP, 28.2%; [ $\gamma$ - $^{16}\text{O}$ ,  $^{18}\text{O}_2$ ]ATP, 38.6%. As the (1*R*,2*S*)-1,2-[1- $^{18}\text{O}$ ]dihydroxy-1,2-diphenylethane was chirally pure, [ $\gamma$ - $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]ATP should all be in the (S)-configuration at phosphorus.

### $^{31}\text{P}$ n.m.r. spectroscopy

Spectra were recorded on a Bruker WH 300 FT spectrometer with quadrature detection at 121.5 MHz.

### Glucokinase-catalysed [ $^{16}\text{O}$ , $^{17}\text{O}$ , $^{18}\text{O}$ ]phosphoryl transfer from adenosine 5'-[ $\gamma$ (S)- $^{16}\text{O}$ , $^{17}\text{O}$ , $^{18}\text{O}$ ]triphosphate to D-glucose

Glucokinase (50 nkat) was added to a solution of the tetrakis-triethylammonium salt of adenosine

5'-[ $\gamma$ (S)- $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]triphosphate (13 mM),  $\text{MgCl}_2$  (14 mM) and D-glucose (0.1 M) in triethanolamine hydrochloride buffer (pH 8.0, 0.05 M, 10 ml) and the solution was kept at 37°C for 4.5 h. The enzyme was then denatured by vigorous agitation of the solution with chloroform. The aqueous layer was degassed, adjusted to the ionic strength of the starting buffer and applied to a DEAE-Sephadex A-25 column (100 ml) in 25 mM-triethylammonium hydrogen carbonate buffer at pH 8.0. The column was eluted with a gradient of triethylammonium hydrogen carbonate buffer (pH 8.0, 25–400 mM) over 24 h at a flow rate of 82 ml/h; 15 min fractions were collected. The fractions containing D-glucose 6-[ $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]phosphate were combined and evaporated under reduced pressure, followed by the addition and evaporation of several volumes of dry methanol to give D-glucose 6-[ $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]phosphate bis-triethylammonium salt (70  $\mu$ mol). {Some unused adenosine 5'-[ $\gamma$ (S)- $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]triphosphate (40  $\mu$ mol) was also recovered from the column.} Treatment with Dowex 50W ( $\text{H}^+$  form) resin in aqueous solution followed by filtration and evaporation under reduced pressure gave D-glucose 6-[ $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]phosphate. Dry dioxan was added, followed by a solution of tri-n-octylamine (70  $\mu$ mol) in dry dioxan. Methanol was added to assist formation of the salt. The solvent was removed and the D-glucose 6-[ $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]phosphate mono-tri-n-octylammonium salt was thoroughly dried by the addition and evaporation of several volumes of dry dioxan.

### Analysis of the chirality at phosphorus of D-glucose 6-[ $^{16}\text{O}$ , $^{17}\text{O}$ , $^{18}\text{O}$ ]phosphate

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

## Results and discussion

D-Glucose 6-[ $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]phosphate obtained from the glucokinase catalysed reaction between adenosine 5'-[ $\gamma$ (S)- $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]triphosphate and D-glucose was cyclized and methylated as previously described (Jarvest *et al.*, 1981). The axial triester is always the predominant diastereoisomer formed, and, as noted previously, the ratio of axial to equatorial triester is rather variable (Jarvest *et al.*, 1981). In this analysis satisfactory spectral data could be obtained for the isotopically labelled axial triesters only, but these gave an unequivocal indication of the stereochemical course of the reaction. The observed relative peak intensities are compared in Table 1 with the expected values for phosphoryl transfer with retention and inversion of configuration. The excellent agreement with the values calculated for inversion, but not with those for retention, shows that the glucokinase-catalysed

Table 1. Comparison of the observed relative peak intensities for the  $^{31}\text{P}$  resonances of the axial triesters with the calculated values expected for glucokinase-catalysed phosphoryl transfer with retention and inversion of configuration at phosphorus

The isotopically labelled esters are obtained by cyclization followed by methylation of the D-glucose 6- $^{16}\text{O},^{17}\text{O},^{18}\text{O}$  phosphate and its isotopomers, derived by the glucokinase-catalysed reaction between adenosine 5'- $\gamma$ (S)- $^{16}\text{O},^{17}\text{O},^{18}\text{O}$  triphosphate and D-glucose. The calculated values are those expected from the known isotopomer composition of ATP. ● =  $^{18}\text{O}$ .

	Peak intensity		
	Observed	Calculated	
		Retention	Inversion
MeO-P=O	0.63	0.74	0.74
Me●-P=O	1.00	0.67	1.00
MeO-P=●	0.69	1.00	0.67
Me●-P=●	0.50	0.46	0.46

phosphoryl transfer between  $\text{MgATP}^{2-}$  and glucose proceeds with inversion of configuration.

This result is essentially the same as that found with yeast hexokinase (Lowe & Potter, 1981), and admits of two interpretations: either the reaction proceeds by an associative 'in-line' mechanism in which there is direct transfer of the phosphoryl group between substrates in the ternary complex, or it proceeds by a dissociative mechanism in which metaphosphate occurs as an intermediate. The former is much the more likely, since for yeast hexokinase the dissociative mechanism has been excluded by positional isotope-exchange measurements (Rose, 1980).

The correspondence in stereochemistry between the reactions catalysed by rat liver glucokinase and by yeast hexokinase lends support to the view that the unusual kinetic properties of glucokinase do not arise from any difference in the chemical mechanisms. Instead, it is reasonable to suppose that all enzymes catalysing the phosphorylation of hexoses follow essentially the same mechanism, but that quantitative differences in rate constants result in a range of types of kinetic behaviour. For two mammalian enzymes, glucokinase and muscle hexokinase type II, isotope-exchange studies have previously shown that the order of addition of substrates and release of products is the same but the step that is rate-limiting for hexokinase type II, the interconversion of ternary complexes, is not rate-limiting for glucokinase (Gregoriou *et al.*, 1981*a,b*).

This unity of chemical mechanism, together with the absence of a sufficient specific difference between glucokinase and other hexokinases (Ureta *et al.*, 1979; Pollard-Knight & Cornish-Bowden, 1982), indicates that glucokinase ought to be regarded as a hexokinase isoenzyme, within the classification EC 2.7.1.1, and that the use of a different name and classification is not justified.

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