The Use of Deoxyfluoro-D-galactopyranoses in a Study of Yeast Galactokinase Specificity

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1. 2-Deoxy-2-fluoro-D-galactose, 3-deoxy-3-fluoro-D-galactose, 4-deoxy-4-fluoro-D-galactose, 6-deoxy-6-fluoro-D-galactose and 2-deoxy-D-*lyxo*-hexose are substrates for yeast galactokinase. 2. The variation in K_m values for the D-hexose derivatives was not associated with a variation in the value of K_m for $MgATP^{2-}$ indicating that the binding of $MgATP^{2-}$ is not modified by the binding of the sugar substrate. 3. Donated H bonds from OH-3, OH-4 and OH-6 and an accepted H bond to OH-2 of the D-hexose are important for the binding of the sugar substrate to galactokinase. 4. Yeast galactokinase exhibits similar kinetics to the galactokinase from *Escherichia coli* and operates by a similar random sequential mechanism. 5. 4-Deoxy-4-fluoro-D-glucose was neither a substrate for nor an inhibitor of yeast galactokinase.

Previous studies from these laboratories illustrated the value of fluorinated analogues of D-glucopyranose and of its 6-phosphate in studies of the mode of action of a number of enzymes (Bessell *et al.*, 1972; Bessell & Thomas, 1973*a,b*). Little is known of the role of -OH groups in the binding of substrate to yeast galactokinase (ATP-D-galactose 1-phosphotransferase; EC 2.7.1.6) an enzyme that is specific for the α anomer of D-galactopyranose (Howard & Heinrich, 1965; Heinrich & Howard, 1966). Since the complete series of deoxyfluoro-D-galactopyranoses is now available (Foster & Westwood, 1973) it became practicable to investigate the effect of substitution of -F for -OH variously in the D-galactose molecule, on the catalytic activity of yeast galactokinase.

Materials and Methods

Galactokinase from galactose-adapted yeast (sp. activity, 16 international units/mg), rabbit muscle pyruvate kinase (ATP-pyruvate phosphotransferase; EC 2.7.1.40) (sp. activity 405 international units/mg), rabbit muscle lactate dehydrogenase (L-lactate-NAD⁺ oxidoreductase; EC 1.1.1.27), 2-deoxy-D-*lyxo*-hexose (2-deoxy-D-galactose) and NADH were purchased from the Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). ATP and phosphoenolpyruvate were from Boehringer Corp. (London) Ltd. (London W.5, U.K.). The deoxyfluoro-D-galactopyranose compounds were synthesized in these laboratories. 2-Deoxy-2-fluoro-D-galactose (Adamson & Marcus, 1972), 3-deoxy-3-fluoro-D-galactose (Brimacombe *et al.*, 1970), 4-deoxy-4-fluoro-D-galactose (Marcus & Westwood,

1971) and 6-deoxy-6-fluoro-D-galactose (Taylor & Kent, 1958).

Assay of galactokinase activity

The assay method employed was basically that described by Heinrich & Howard (1966). Initial-rate measurements were carried out with a Cary model 16 KC kinetic spectrophotometer, the output of which was connected to a Varian strip chart recorder model G-2510. The full-scale deflexion of the recorder was set to correspond to a ΔE_{340} of 0.01 or 0.02 and the cell compartment was thermostatically maintained at 30°C. All reactions were carried out in 0.05M-sodium phosphate buffer (pH 7.0) containing 0.08M-KCl, 0.002M-MgCl₂, 0.001M-phosphoenolpyruvate and NADH (0.08mg/ml). The amounts of auxiliary enzymes needed to attain a steady state after a short lag-time (6s) were those suggested by McClure (1969) with pyruvate kinase (7 units/ml) and lactate dehydrogenase (5 units/ml) in a total volume of 1 ml. Reactions were initiated by the addition of 0.1 ml of galactokinase.

Data processing

Values of K_a , K_b , K_{ia} and V_{max} were obtained by using a computer program written by Cleland (1963) and modified for use on the London University CDC 6600 computer (see Bessell *et al.*, 1972). K_a is the limiting K_m value for the D-galactose derivative, K_b the limiting K_m value for $MgATP^{2-}$ and K_{ia} the dissociation constant for the D-galactose derivative.

Paper chromatography

Descending paper chromatography of reaction mixtures was carried out as described by Kent & Wright (1972) on Whatman no. 1 paper with a 2-methoxyethanol-pyridine-NH₃-water (5:2:1:2, by vol.) mixture as the developing solvent. Phosphate esters were identified by using a molybdate spray reagent (Bandurski & Axelrod, 1951).

Results and Discussion

All the compounds in the series of deoxyfluoro-D-galactopyranoses were used as substrates for yeast

galactokinase and the experimentally determined kinetic constants are shown in Table 1. All the double-reciprocal plots were linear and were consistent with the plots shown in Figs. 1 and 2 for galactose as the variable substrate. In the case of 3-deoxy-3-fluoro-D-galactose, however, owing to insufficient material only one concentration of MgATP²⁻ was used.

The results obtained fit the equation:

$$v = \frac{V[AB]}{K_{ia}K_b + K_a[B] + K_i[A] + [AB]}$$

and indicate a sequential mechanism. Similar initial-velocity patterns to those seen in Figs. 1 and 2 were

Table 1. Kinetic constants for yeast galactokinase

Values are means $\pm$ S.E.M. Values in parentheses indicate the number of concentrations of MgATP²⁻ and sugar used.

Compound	K_a (mM)	K_{ia} (mM)	K_b (mM)	Relative V_{max}	Concentration range for MgATP ²⁻ (mM)	Concentration range for sugar (mM)	No. of experimental points
D-Galactose	0.9 ± 0.4	1.6 ± 0.6	1.1 ± 0.3	1.0	0.31–2.5 (4)	0.31–5.0 (5)	20
2-Deoxy-D-galactose	108 ± 26.5	52 ± 12	2.4 ± 0.6	0.73	0.31–2.5 (4)	12.5–100 (4)	16
2-Deoxy-2-fluoro-D-galactose	5.6 ± 2.3	8.3 ± 4.2	1.0 ± 0.5	0.24	0.31–2.5 (4)	3.5–28 (4)	16
3-Deoxy-3-fluoro-D-galactose*	60	—	—	0.10	2.5 (1)	5–25 (4)	4
4-Deoxy-4-fluoro-D-galactose	192.5 ± 71	96.5 ± 37	2.1 ± 0.8	0.095	0.31–2.5 (4)	12.5–200 (5)	20
6-Deoxy-6-fluoro-D-galactose	55 ± 38	—	1.0 ± 0.4	0.08	0.31–2.5 (4)	12.5–100 (3)	12

* Approximate values only owing to insufficient material.

Fig. 1. Initial-velocity pattern with galactose as the varied substrate

MgATP²⁻ concentrations (mM): ●, 2.5; ○, 1.25; △, 0.625; ▲, 0.3125. Initial velocities are expressed as $\Delta E_{340}/\text{min}$. For experimental details see the text. Lines are computer-determined 'best fits'.

Fig. 2. Initial-velocity pattern with MgATP²⁻ as the varied substrate

Galactose concentrations (mM): ○, 5.0; ●, 2.5; △, 1.25; ▲, 0.625; ■, 0.3125. Initial velocities are expressed as $\Delta E_{340}/\text{min}$. For experimental details see the text. Lines are computer-determined 'best fits'.

reported by Gulbinsky & Cleland (1968) for the galactokinase from *Escherichia coli*. These patterns differ from those obtained with yeast hexokinase (ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1) (Lueck *et al.*, 1973) in that the point of intersection lies above the abscissa, whereas in the case of hexokinase the intersection occurs on this axis. Gulbinsky & Cleland (1968) also produced kinetic evidence that the mechanism of *E. coli* galactokinase is in fact of the random sequential type. This also appears to be the case for yeast galactokinase. Unlike the hexokinase situation (Bessell *et al.*, 1972), K_s does not vary with a change in the sugar substrate indicating that the 'induced fit' hypothesis that has been suggested for hexokinase (Delafuente *et al.*, 1970), and which involves the modification of the MgATP^{2-} binding site by the initial binding of the sugar substrate, cannot be postulated in the case of the binding of MgATP^{2-} to galactokinase.

Kent & Wright (1972) first showed that 6-deoxy-6-fluoro-D-galactose was a substrate for yeast galactokinase though kinetic data were not presented. They synthesized 6-deoxy-6-fluoro-D-galactopyranosyl phosphate chemically and showed that it was the product of the phosphorylation of 6-deoxy-6-fluoro-D-galactose by galactokinase. By using a similar procedure involving paper chromatography the present work shows that a phosphate-containing product with a mobility similar to that of a sugar 1-phosphate was produced during the phosphorylation of 2-deoxy-2-fluoro-D-galactose by galactokinase and MgATP^{2-} . Attempts to detect 1-phosphates produced with other fluorogalactoses were not successful as only a small percentage of the total sugar was phosphorylated at the enzyme concentration used in the assay.

Galactokinase will phosphorylate the sugar substrates to form the sugar pyranose-1-phosphate and it is therefore somewhat surprising that the values obtained for K_s and V_{max} for the deoxyfluoro-D-galactopyranoses were qualitatively similar to those obtained for the deoxyfluoro-D-glucopyranoses with yeast hexokinase (Bessell *et al.*, 1972) as the product of glucose phosphorylation by hexokinase is the 6-phosphate analogue. However, unlike hexokinase OH-2 does appear to play a role in the binding of the sugar substrate to galactokinase. The low value for K_s found with 2-deoxy-2-fluoro-D-galactose as the sugar substrate compared with the high value obtained when 2-deoxygalactose was the sugar substrate indicates that the role of OH-2 is to accept a H bond from the enzyme. (F can accept but not donate a H bond.) Thus talose (the 2-epimer of D-galactose) and 2-amino-2-deoxy-D-galactose (galactosamine) have also been reported as substrates for the enzyme (Alvarado, 1960), although some doubt exists as to whether galactosamine is in fact phosphorylated (Heinrich, 1964). All OH-3, OH-4 and OH-6 groups seem to have similar roles in H-bond donation as

substitution of these groups by F has the effect of considerably increasing the value of K_s . Thus whereas OH-3, OH-4 and OH-6 are involved in the binding to galactokinase and hexokinase as H-bond donors there is an additional H bond accepted at OH-2 in the case of galactokinase. As D-galactose exists in its pyranose form in aqueous solution (Angyal, 1969) and the product of the reaction is also in the pyranose form, then it is extremely likely that D-galactose will bind to the enzyme in this configuration. Similarly D-glucose probably also binds to hexokinase as a pyranose sugar although an intermediate glucose-enzyme complex involving the acyclic form of D-glucose has been postulated (Bessell, 1973).

The specificity of galactokinase for the D-galacto configuration was borne out by the fact that 4-deoxy-4-fluoro-D-glucopyranose was neither a substrate nor an inhibitor (at concentrations up to 50mm) for yeast galactokinase. This shows that not only can galactokinase distinguish between an axial OH at C-4 (D-galactopyranose in the C1 conformation) and an equatorial OH at C-4 (D-glucopyranose in the C1 conformation) but it can also distinguish between an axial and an equatorial F atom at C-4.

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