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THE SUBSTRATE SPECIFICITY OF YEAST HEXOKINASE: REACTION WITH D-ARABINOSE OXIME

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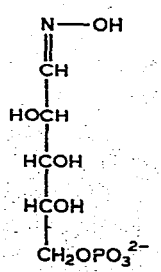
ABSTRACT

By chromatography, electrophoresis, n.m.r. spectroscopy, and spectrophotometric assay, it has been shown that D-arabinose oxime acts as a weak substrate for yeast hexokinase. The enzyme-catalysed phosphorylation of the oxime, which exists as a mixture of *E* (80%) and *Z* (20%) acyclic forms in solution at equilibrium, is proposed to proceed *via* the transient formation of a furanoid species. Weak substrate-activity was also observed with 4-deoxy-D-*xylo*-hexose, but not with 5-deoxy-D-*xylo*-hexose. The relation of these and previous results concerning the carbohydrate-substrate specificity of yeast hexokinase in solution to X-ray crystallographic studies is discussed.

INTRODUCTION

In an effort to prepare D-arabinose oxime 5-phosphate (**1**), a reaction-intermediate analogue for the reaction catalysed by phosphoglucose isomerase, we have investigated the interaction of D-arabinose oxime with yeast hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) and MgATP, and found that phosphoryl transfer to the primary alcohol function of the oxime occurs readily. This result was somewhat surprising, because no acyclic substrate of hexokinase has been reported, and D-arabinose oxime has been shown to adopt acyclic *E* and *Z* structures in the solid state and in aqueous solution[1,2]. We now report on the evidence for catalysis of the phosphorylation of D-arabinose oxime by hexokinase, and also on experiments with 4- and 5-deoxy-D-*xylo*-hexoses as possible substrates.



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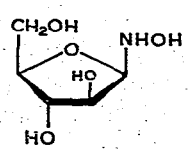

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RESULTS

Paper chromatography. — Analysis of a solution containing D-arabinose oxime, ATP, and magnesium ions, 24 h after the addition of hexokinase, showed the presence of a component with a positive response to silver nitrate and acid molybdate reagents and with a mobility equal to that of D-glucose 6-phosphate. A component of similar mobility was observed when 4-deoxy-D-*xylo*-hexose was the sugar component in the reaction mixture, but not when 5-deoxy-D-*xylo*-hexose was present.

Paper electrophoresis. — Analysis of a reaction mixture containing D-arabinose oxime and MgATP, 24 h after the addition of hexokinase, revealed a new component having a positive response to the silver nitrate reagent and an electrophoretic mobility equal to that of D-glucose 6-phosphate. A similar result was obtained with 4-deoxy-D-*xylo*-hexose as the carbohydrate substrate, but the reaction had not proceeded to completion; no reaction was apparent with 5-deoxy-D-*xylo*-hexose. The oximes of D-ribose, D-mannose, D-galactose, and D-glucose also appeared to act as substrates in the phosphorylation reaction, but less readily than did D-arabinose oxime.

^{1}H-N.m.r. spectroscopy. — The 360-MHz ^{1}H-n.m.r. spectrum of a solution in D_2O containing D-arabinose oxime and MgATP showed the expected[1] signals from the *E* and *Z* forms of the oxime, and other resonances, particularly a doublet at δ 5.99 and singlets at 7.99 and 8.30 attributable[3] to H-1′, H-2, and H-8 of ATP; 14 h after the addition of hexokinase, the singlets at δ 7.99 and 8.30 had decreased in intensity, new singlets of about equal intensity were apparent at δ 8.36 and 7.98, and the signal at δ 5.99 had become more complex. These changes are consistent with the conversion of ATP into ADP. The well-resolved doublets arising from H-1 of the *E* and *Z* forms of the oxime were not altered in shift or splitting or intensity during the experiment. However, signals assigned to H-5a,5b shifted downfield by 0.22 p.p.m. and became more complex, changes which are consistent[4] with the introduction of a phosphoryl group at O-5.

The 220-MHz ^{1}H-n.m.r. spectrum of D-arabinose oxime monophosphate dilithium salt, prepared by carrying out the hexokinase reaction on a larger scale (see Experimental), showed two doublets at δ 6.99 and 6.36 of relative intensity 83:17, arising from H-1 of the *E* and *Z* forms of the oxime. The signals assigned to H-5 were more complex, and were shifted downfield by 0.11 p.p.m. compared to those of non-phosphorylated D-arabinose oxime.

^{31}P-N.m.r. spectroscopy. — The ^{31}P-n.m.r. spectrum of D-arabinose oxime and MgATP showed the expected[5] signals for the α, β, and γ phosphorus atoms of ATP at 10.39(d), 19.53(t), and 6.20(d) p.p.m. upfield from external H_3PO_4 (85% w/v in water). In the spectrum taken 3.5 h after the addition of hexokinase, the signals due to ATP had diminished in intensity and new signals were apparent at 10.15(d) and 6.94(d) p.p.m. upfield, and 2.48(s) p.p.m. downfield, from H_3PO_4. These changes are consistent with the formation of ADP and a monophosphoric ester from ATP and D-arabinose oxime. The proton-decoupled ^{31}P-n.m.r. spectrum of the enzymically synthesised and isolated D-arabinose oxime 5-phosphate dilithium salt gave single

lines at δ 4.54, 3.67, and 2.40 downfield from 85% H_3PO_4 of relative intensity 81:17:2. The undecoupled spectrum showed broad triplets centred at δ 4.54 and 3.67, which were assigned to phosphorus nuclei coupled to H-5a,5b in the *E* and *Z* oxime phosphate, respectively.

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS[a] OF D-ARABINOSE OXIMES AND D-ARABINOSE OXIME 5-PHOSPHATES

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*
E-D-Arabinose oxime	155.1	71.1	74.9	73.4	65.4
E-D-Arabinose oxime 5-phosphate	255.6	71.2	74.2	72.8[b]	67.7[c]
Z-D-Arabinose oxime	156.0	67.1	74.1	73.4	65.4
Z-D-Arabinose oxime 5-phosphate	156.5	67.1	73.6	72.8[b]	67.7[c]

[a]P.p.m. relative to internal TSP. [b]$^3J_{^{31}P,^{13}C}$ = 5.9 Hz (d). [c]$^2J_{^{31}P,^{13}C}$ = 4.4 Hz (d).

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. parameters for D-arabinose oxime and the enzymically synthesised and isolated 5-phosphate dilithium salt are presented in Table I. The signal assignments for D-arabinose oxime were made by off-resonance decoupling and by comparison[6] with ^{13}C-n.m.r. data[7] for D-arabinitol and 1-deoxy-D-arabinitol. The pertinent observations are the downfield shifts of 2.3 p.p.m. for the C-5 resonances of the phosphorylated oxime and the two-bond ^{31}P–^{13}C and three-bond ^{31}P–^{13}C couplings of 4.4 and 5.9 Hz, respectively. These observations are consistent[8–11] with phosphorylation at HO-5. The relative intensities of the C-1 resonances corresponded to a ratio of *E* to *Z* forms of 81.5:18.5.

Coupled enzyme assay. — The initial rates of reactions of MgATP with both fresh (100% *Z* form) and equilibrated (20% *Z*, 80% *E* forms) solutions of D-arabinose oxime catalysed by hexokinase gave linear Lineweaver–Burk plots when plotted in reciprocal form. The apparent K_m and V_{max} values are given in Table II. At substrate concentrations of 10mM, the oximes (equilibrated solutions) of D-ribose, D-mannose, D-glucose, and D-galactose displayed initial rates of reaction 12–120 times lower than that (1.35 μmol.min^{-1}.mg of protein^{-1}) of D-arabinose oxime; 4-deoxy-D-*xylo*-hexose reacted 40 times more slowly and 5-deoxy-D-*xylo*-hexose showed no measurable reaction.

TABLE II

APPARENT MICHAELIS–MENTEN PARAMETERS FOR THE PHOSPHORYLATION OF D-ARABINOSE OXIMES CATALYSED BY YEAST HEXOKINASE

Substrate	K_m *(mM)*	V_{max} *μmol.min^{-1}.mg of protein^{-1}*
Z-D-Arabinose oxime	180	8.5
Z (20%) + *E* (80%)-D-Arabinose oxime	97	3.1

DISCUSSION

The substrate specificity of yeast hexokinase may be conveniently examined by considering the values of the phosphorylation coefficient, namely, V/K_m for different substrates[12]. Some phosphorylation coefficients, relative to the values obtained for equilibrated D-glucose, are given in Tables III and IV. Small differences between phosphorylation coefficients are of little significance, because kinetic data for hexokinases are somewhat variable due to dependencies on ion and coenzyme

TABLE III

PYRANOID SUBSTRATES OF YEAST HEXOKINASE

Compound	*Phosphorylation coefficient* V/K_m *relative to* D-*glucose*
α-D-Glucose[13]	1.15
β-D-Glucose[13]	0.95
D-Mannose[12]	1.50
2-Deoxy-D-*arabino*-hexose[12]	0.30
2-Amino-2-deoxy-D-glucose[12]	0.05
D-*arabino*-2-Hexosulose[12]	1.00
2-Deoxy-2-fluoro-D-glucose[14]	0.45
2-Deoxy-2-fluoro-D-mannose[14]	0.35
2-Deoxy-2,2-difluoro-D-*arabino*-hexose[14]	0.70
2-Chloro-2-deoxy-D-glucose[14]	0.044
D-Allose[13]	$\sim 10^{-4}$
3-Deoxy-3-fluoro-D-glucose[14]	2.4×10^{-4}
2-Amino-2-deoxy-α-D-glucose[30]	0.1
2-Amino-2-deoxy-β-D-glucose[30]	0.02
D-Galactose[12]	4×10^{-6}
4-Deoxy-4-fluoro-D-glucose[14]	2×10^{-4}
4-Deoxy-D-*xylo*-hexose	$\sim 2 \times 10^{-6}$
D-*threo*-2,5-Hexodiulose[15]	0.14
5-Thio-D-glucose[16]	0.032
1,5-Anhydro-D-glucitol[12]	3.3×10^{-4}

TABLE IV

FURANOID SUBSTRATES OF YEAST HEXOKINASE

Compound	*Phosphorylation coefficient* V/K_m *relative to* D-*glucose*
D-Fructofuranose[17,18]	1.2
D-Arabinofuranose[a]	2.9×10^{-4}
1-Deoxy-D-fructofuranose[19]	2.6×10^{-5}
2,5-Anhydro-D-mannose[18]	0.35
2,5-Anhydro-D-mannitol[18]	0.024
2,5-Anhydro-D-glucitol[18]	2.4×10^{-3}
D-Arabinose oxime (all species in equilibrated solution)	3.3×10^{-5}

[a]These measurements were made with the technical assistance of Mr. B. M. Cockerill.

concentrations[20]; also, the different isoenzyme forms possess different kinetic properties[21]. However, in spite of these limitations, it is clear from previous studies (see Tables III and IV) that yeast hexokinase is highly specific for cyclic pyranoid or furanoid hexose derivatives having the D-*arabino* configuration at C-3, C-4, and C-5. The enzyme shows little discrimination between the anomeric forms of D-glucopyranose[13], and the phosphorylation coefficients, other than those for 2-amino-2-deoxy-D-glucose, in Table III have been calculated without regard to possible differences between anomers.

In principle, the majority of the hexose derivatives listed in Table III could react at the active site of hexokinase in the pyranoid, furanoid, or acyclic forms, but previous results with D-glucitol[12], a non-substrate and non-inhibitor, 5-deoxy-D-*xylo*-hexose 6-phosphate, a non-inhibitor[31], and 1,5-anhydro-D-glucitol[12], a poor substrate, suggest that the pyranoid forms are the active species. This view is reinforced by the lack of reactivity of 5-deoxy-D-*xylo*-hexose, which cannot adopt a pyranoid form, and the small but definite reactivity of 4-deoxy-D-*xylo*-hexose, which cannot adopt a furanoid form, observed in the present work. The phosphorylation coefficients for the first three compounds in Table IV have been calculated by using the proportions of α and β furanoid forms present at equilibrium (data of Angyal and his co-workers[22]). The results for the anhydro compounds in Table IV strongly indicate that the enzyme shows a higher specificity for the β rather than the α furanoid form; if so, this would correspond to slightly higher values of the phosphorylation coefficients for the first three compounds in Table IV.

Fromm and co-workers[20] pointed out that the hydroxyl substituents on carbon atoms 1, 3, 4, and 6 of β-D-glucopyranose in the $^4C_1$ conformation can be oriented in approximately the same way as the hydroxyl groups on carbon atoms 2, 3, 4, and 6 of β-D-fructofuranose. Indeed, examination of molecular models shows that HO-2 of D-mannopyranose and β-D-fructopyranose can take up a similar orientation, and this is consistent with the higher phosphorylation coefficients of these compounds in comparison to D-glucopyranose ($^4C_1$) in which HO-2 is equatorial. A structural basis for the solution specificity of yeast hexokinase has been provided by the X-ray crystallographic studies of the binding of glucose and "*o*-toluoylglucosamine" carried out by Steitz and co-workers[23,24]. They observed a multivalent network of hydroxyl groups of the glucose ring, which, as they noted, is consistent with the extreme sensitivity shown by the enzyme to substitution at these points. Indeed, the low phosphorylation coefficients of the 3- and 4-deoxyfluoroglucoses[14] suggest that hydrogen-bond donor interactions as well as hydrogen-bond acceptor interactions must be important[31]. The X-ray crystallographic analysis did not clearly reveal the anomeric character of the bound glucose, but solution studies[13] imply that both α- and β-forms can be bound. Examination of molecular models shows that HO-1 of β-D-fructopyranose can adopt orientations corresponding quite closely to those of HO-1 in α- or β-D-glucose by rotation about the fructose C-1–C-2 bond. The importance of interactions with the enzyme of a hydroxyl group at C-1 in one of these orientations is clearly shown by the low phosphorylation coefficients (Tables III

and IV) of 1,5-anhydro-D-glucitol, D-arabinofuranose, 1-deoxy-D-fructofuranose, and 2,5-anhydro-D-glucitol.

From the above considerations, it may be concluded that the observed phosphorylation of D-arabinose oxime catalysed by hexokinase must proceed *via* a furanoid form of the compound, preferably of β-anomeric configuration (**2**). This structure is an N-1 analogue of 2,5-anhydro-D-mannitol, which has a phosphorylation coefficient of 0.024 relative to D-glucopyranose. The true phosphorylation coefficient of **2** and the alternative anomeric form cannot be calculated, since the proportions of these forms present in solution are not known. However, the results imply their presence and the possibility that the mutarotation of *Z*-D-arabinose oxime[6] proceeds through such cyclic forms. Further, there are well-established examples of enzymes, *e.g.*, muscle aldolase[25] and triose phosphate isomerase[26], which act exclusively on isomeric forms that are present in minor amount in equilibrium in solution, and this must also be the case for hexokinase action on D-arabinose. The proposition of such cyclic oxime species is not without precedent, since D-glucose oxime is known[1,2] to adopt the β-pyranoid form in the solid state and in solution (to some extent). The fact that the hexokinase-catalysed phosphorylations of the *Z* form and an equilibrium mixture of D-arabinose oximes at equal, total concentrations proceed at different rates implies that the *Z* form generates a higher concentration of the reactive furanoid species than does the equilibrium mixture.

EXPERIMENTAL

General. — The oximes of D-arabinose, D-glucose, D-ribose, D-mannose, and D-galactose were prepared as described previously[1]. 4-Deoxy-D-*xylo*-hexose (as the methyl α-D-hexoside) was kindly donated by Dr. D. Gibson; 5-deoxy-D-*xylo*-hexose (as the 1,2-*O*-isopropylidene derivative) was kindly donated by Professor W. G. Overend and Dr. E. J. Hedgley. The yeast hexokinase used was Sigma Type F300 (specific activity 7.75 μkat per mg of protein).

Paper chromatography was performed by the descending technique on Whatman No. 1 and 3MM paper with di-isopropyl ether–formic acid (3:2) as mobile phase[27]. Detection was effected with silver nitrate, ferric chloride–formalin[28] (for oximes), or acidic ammonium molybdate[27] (for phosphate esters) reagents.

Paper electrophoresis was performed on Whatman 3MM paper with 0.5M phosphate buffer (pH 7.0), using a Shandon high-voltage electrophoresis apparatus L24 for 1.6 h at 3.0 kV. Components were detected with silver nitrate reagent.

^{1}H-N.m.r. spectra were recorded on Varian EM360, HA100, HR220 (PCMU), or Bruker HX 360 (courtesy of Professor H. J. C. Berendson and Dr. R. Kaptein, University of Groningen, Netherlands) spectrometers at ambient temperature (~28°). In the enzyme phosphorylation experiments at 360 MHz, equimolar (0.2M) amounts of D-arabinose oxime and MgATP were used and 4.7 μkat of hexokinase were added; chemical shifts were recorded relative to internal DSS.

^{31}P-N.m.r. spectra were recorded with a Bruker HX90E spectrometer operating

at 36.431 MHz (King's College, University of London). Solutions of D-arabinose oxime and MgATP were 0.5M in D_2O, and chemical shifts were recorded relative to external 85% H_3PO_4 in H_2O.

^{13}C-N.m.r. spectra were recorded for 0.5M solutions in D_2O using Bruker HX90E spectrometers (PCMU or King's College, University of London) operating at 22.63 MHz; chemical shifts were recorded relative to internal TSP.

Coupled enzyme assay. — Initial rates of phosphorylation catalysed by yeast hexokinase were measured by using the coupled enzyme system of Jaworck *et al.*[29]. The following reagents were added in sequence to give a final volume of 3 cm^3: 0.5M tris(hydroxymethyl)aminomethane buffer (pH 7.5; 1.8 or 1.9 cm^3), phosphoenol pyruvate (19.5mM) and KCl (1.3M) in Tris buffer (0.2 cm^3), NADH disodium salt (12mM in 5% w/v sodium carbonate, 0.05 cm^3), rabbit-muscle pyruvate kinase (1.5 μkat/cm^3 of Tris buffer, 0.05 cm^3), pig-heart lactate dehydrogenase isoenzyme H_4 (18.0 μkat/cm^3 of Tris buffer, 0.05 cm^3), ATP (15mM) and $MgCl_2 \cdot 6H_2O$ (176mM) in Tris buffer (0.15 cm^3), substrate solution (0.5 cm^3 in Tris buffer), and yeast hexokinase (0.33–0.83 μkat per cm^3) and bovine serum albumin (1 mg per cm^3) in Tris buffer (0.1 or 0.2 cm^3). This mixture was placed in a jacketed 1-cm cuvette, and the absorbance was monitored at 340 nm and 25°, against a blank containing all of the reagents except yeast hexokinase, using a Pye Unicam SP1800 spectrophotometer. A blank correction was made by monitoring the absorbance of a solution containing all reagents except sugar substrate against a double blank containing no substrate or hexokinase. Under these conditions, less than 1% of the substrate was phosphorylated and ~10% mutarotation of *Z*-arabinose oxime occurred (observed polarimetrically).

D-Arabinose oxime 5-phosphate. — D-Arabinose oxime 5-phosphate was prepared by using the ATP-regenerating system of Avigad and Englard[15]. The pH of a solution of tris(hydroxymethyl)aminomethane (5 mmol) in deionised water (80 cm^3) was adjusted to 7.0 with M hydrochloric acid. To this solution were added magnesium chloride (0.2 mmol) and adenosine 5′-triphosphate disodium salt (0.15 mmol), and the pH was adjusted to 7.0 with M sodium hydroxide. Phosphoenol pyruvic acid monocyclohexylamine salt (3.98 mmol), potassium chloride (4 mmol), and pyruvate kinase (from rabbit muscle, 4.17 μkat) were added and the pH was readjusted to 7.0 with M sodium hydroxide. To the slowly stirred solution was added D-arabinose oxime (4.82 mmol) followed by yeast hexokinase (Sigma F300, 40 μkat). The reaction was monitored by paper chromatography and electrophoresis. After 24 h, when no D-arabinose oxime could be detected, the solution was filtered and the pH adjusted to 4.0 with hydrochloric acid. This solution was freeze-dried, and a solution of the residue in deionised water (20 cm^3) was extracted with ether (5 × 100 cm^3). To the aqueous layer were added lithium carbonate (4.15 mmol) and cold ethanol (60 cm^3). The precipitate obtained after refrigeration at −5° overnight was collected by centrifugation and dried in a desiccator at 5° to give the title compound (800 mg, 66%). Paper chromatography and electrophoresis showed a single spot.

Anal. Calc. for $C_5H_{10}Li_2NO_8P \cdot 2H_2O$: C, 20.5; H, 4.8; N, 4.7. Found: C, 20.4; H, 4.8; N, 4.4.

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