The Deoxyfluoro-D-glucopyranose 6-Phosphates and their Effect on Yeast Glucose Phosphate Isomerase

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1. The deoxyfluoro-D-glucopyranose 6-phosphates were prepared from the corresponding deoxyfluoro-D-glucoses and ATP by using hexokinase. 2. 3-Deoxy-3-fluoro- and 4-deoxy-4-fluoro-D-glucose 6-phosphate were substrates for glucose phosphate isomerase, and in addition the products of this reaction, 3-deoxy-3-fluoro- and 4-deoxy-4-fluoro-D-fructose 6-phosphate respectively, were good substrates for phosphofructokinase. 3. Some C-2-substituted derivatives of D-glucose 6-phosphate were found to be competitive inhibitors of glucose phosphate isomerase. 4. The possible role of the hydroxyl groups in the binding of D-glucose 6-phosphate to glucose phosphate isomerase is discussed.

Considerable information on the mechanism of action of glucose phosphate isomerase (D-glucose 6-phosphate ketol-isomerase, EC 5.3.1.9) has been obtained (Rose & O'Connell, 1961; Dyson & Noltmann, 1968). Dyson & Noltmann (1968) have suggested a mechanism in which D-glucose 6-phosphate binds initially in its α -pyranose form and then ring opening to the acyclic form occurs. Ring opening is catalysed by a protonated ϵ -amino group of a lysine residue in the active site, which protonates the ring oxygen of the sugar. The hydrogen on C-2 is then transferred to C-1 (via an enediol anion intermediate) by the basic imidazole nitrogen of a histidine residue and finally, ring closure to the furanose form of D-fructose 6-phosphate occurs.

However, little is known about the role and importance of the hydroxyl groups of the sugar phosphate in the binding of the substrate to glucose phosphate isomerase, and substrate-specificity studies have been limited. Only the α -anomer of D-glucopyranose 6-phosphate is a substrate for the enzyme (Salas *et al.*, 1965) and D-mannose 6-phosphate is not a substrate. In contrast, a large number of competitive inhibitors are known (Salas *et al.*, 1965; Parr, 1956; Grazi *et al.*, 1960) and the inhibitors with the greatest affinity for the enzyme are sugar phosphates which can exist only in an acyclic form.

The deoxyfluoro-D-glucose 6-phosphates were therefore prepared to gain some information on the role of the sugar hydroxyl groups in the isomerization reaction.

Experimental

Glucose phosphate isomerase (from yeast, 350 units/mg), phosphofructokinase (from rabbit muscle, 60 units/mg) and ATP were purchased from

Boehringer Corp. (London) Ltd., London W.5, U.K. Optical rotations were determined for aqueous solutions (10 cm light-path) by using a Perkin-Elmer 141 polarimeter. ^{19}F and ^1H n.m.r. spectra were determined by using a Perkin-Elmer R-10 spectrometer. ^1H n.m.r. spectra were recorded at 60 MHz and ^{19}F n.m.r. spectra were recorded at 56.5 MHz. Paper electrophoresis was performed with a Shandon Universal electrophoresis unit (Shandon Scientific Co. Ltd., London N.W.10, U.K.), by using Whatman no. 1 paper, a borate buffer, pH 10 (Foster, 1957) and detection with alkaline AgNO_3 (Trevelyan *et al.*, 1950). The electrophoresis was done for 1 h at 300 V and at 25°C. Analytical Dowex resins were purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

The derivatives of D-glucose 6-phosphate were prepared from the corresponding D-glucose derivatives and ATP by using hexokinase. The deoxyfluoro-D-glucoses were synthesized in these laboratories (see Bessell *et al.*, 1972).

Synthesis of 2-deoxy-2-fluoro-D-glucose 6-phosphate (disodium salt)

2-Deoxy-2-fluoro-D-glucose (Adamson *et al.*, 1970) (250 mg), ATP (disodium salt; 900 mg) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (610 mg) were dissolved in 0.1 M-Tris-HCl buffer, pH 7.5 (30 ml). The pH was adjusted to 8.0 with 1 M-NaOH solution, and yeast hexokinase (400 units) was added. The pH was maintained at pH 8.0 by the addition of aqueous NaOH. The reaction was left overnight at room temperature to ensure complete reaction. The solution was then added to a column (80 cm $\times$ 2.5 cm) of Dowex resin (AG1; X4; borate form; 200–400 mesh) (Lefebvre

et al., 1964). The product of the reaction, 2-deoxy-2-fluoro-D-glucose 6-phosphate, was eluted with a linear gradient of triethylammonium tetraborate (1 litre, 0.1–0.4M). Concentration of the appropriate fractions under reduced pressure and subsequent co-concentration with methanol to remove borate gave the triethylammonium salt of 2-deoxy-2-fluoro-D-glucose 6-phosphate. This was dissolved in water (10ml) and applied to a column (20cm×1cm) of Dowex resin (AG 50W; X8; Na⁺ form; 200–400 mesh). Elution with water and freeze-drying of the appropriate fractions gave the disodium salt of 2-deoxy-2-fluoro-D-glucose 6-phosphate (190mg) as an amorphous solid, $[\alpha]_D^{25} +23^\circ$ (c 0.6 in water).

The following compounds were also prepared as disodium salts in the same manner: 3-deoxy-3-fluoro-D-glucose 6-phosphate, $[\alpha]_D^{25} +11^\circ$ (c 0.8 in water); 4-deoxy-4-fluoro-D-glucose 6-phosphate, $[\alpha]_D^{25} +20^\circ$ (c 0.6 in water); 2-chloro-2-deoxy-D-glucose 6-phosphate, $[\alpha]_D^{25} +10^\circ$ (c 1.0 in water); 2-deoxy-D-arabinohexose 6-phosphate, (2-deoxy-D-glucose 6-phosphate) $[\alpha]_D^{25} +11^\circ$ (c 1.5 in water); the sample obtained from Sigma (London) Chemical Co., London S.W.6, U.K., had $[\alpha]_D^{25} +12^\circ$ (c 0.3 in water). 2-Amino-2-deoxy-D-glucose 6-phosphate (disodium salt) [D-glucosamine 6-phosphate (disodium salt)] and D-glucose 6-phosphate (disodium salt) were purchased from Sigma (London) Chemical Co.

Confirmation of structure, apart from that assumed from an enzymic synthesis, was carried out by using ¹⁹F n.m.r. spectroscopy and field-desorption mass spectrometry (H. D. Beckey, E. M. Bessell, A. B. Foster, M. Jarman, H.-R. Shulten & J. H. Westwood, unpublished work). ¹⁹F n.m.r. spectroscopy is a sensitive technique for obtaining not only confirmation of structure but also evidence for homogeneity, i.e. impurities (>5%) containing fluorine can be detected. All the deoxyfluoro-D-glucose 6-phosphates were shown to be homogeneous in this way,

and the similarity of the spectra (determined by using aqueous solutions) to those of the corresponding deoxyfluoro-D-glucoses (Barford *et al.*, 1971; Phillips & Wray, 1971) gave confirmation of structure. A comparison between the ¹H–¹⁹F coupling constants of the deoxyfluoro-D-glucose 6-phosphates and those of the deoxyfluoro-D-glucoses (Phillips & Wray, 1971) is given in Table 1.

¹H n.m.r. spectroscopy was also useful because the ¹H n.m.r. spectra (in ²H₂O, internal standard methyl cyanide) of the triethylammonium salts of the deoxyfluoro-D-glucose 6-phosphates showed resonances for the triethylammonium ion at δ 1.0 (triplet, *J* 7 Hz, CH₃–) and δ 2.1 (quartet, *J* 7 Hz, –CH₂–N⁺). These resonances were absent in the ¹H n.m.r. spectra of the disodium salts.

The homogeneity of these compounds was also shown by paper electrophoresis (see above); no starting material could be detected. Their mobilities were identical with that of D-glucose 6-phosphate. Clear separations from any remaining starting material were obtained by using the Dowex resin (borate form) described above. Only with 3-deoxy-3-fluoro-D-glucose 6-phosphate was there any overlap of the fractions with those of 3-deoxy-3-fluoro-D-glucose. The greater affinity of 3-deoxy-3-fluoro-D-glucose for borate than the other D-glucose derivatives has been shown by paper electrophoresis (Bessell, 1970). Also 3-deoxy-3-fluoro-D-glucose 6-phosphate was not detected as easily as the other phosphates with alkaline AgNO₃.

Initial-rate measurements

The forward reaction of glucose phosphate isomerase was assayed by coupling to phosphofructokinase. The Cresol Red method described by Darrow & Colowick (1962) for assaying hexokinase activity was used to measure the rate of proton release. This

Table 1. ¹H–¹⁹F coupling constants (in Hz) of the deoxyfluoro-D-glucose 6-phosphates in ²H₂O

For experimental details see the text. The relative amount of each anomer was determined by using ¹⁹F n.m.r. spectra. In all three compounds approximately equal amounts were present. The coupling constants (*J*) in parentheses are those quoted by Phillips & Wray (1971) for the non-phosphorylated compounds. ($\phi_\alpha - \phi_\beta$) is the difference in chemical shift between the ¹⁹F resonance for the α anomer and the ¹⁹F resonance for the β anomer. The values of ($\phi_\alpha - \phi_\beta$) in parentheses are those for the non-phosphorylated compounds [Adamson *et al.* (1970); Barford *et al.* (1971)].

Compound	Anomeric form	<i>J</i> _{H-1,F}	<i>J</i> _{H-2,F}	<i>J</i> _{H-3,F}	<i>J</i> _{H-4,F}	($\phi_\alpha - \phi_\beta$)
2-Deoxy-2-fluoro-D-glucose 6-phosphate	α	<0.5 (<0.5)	49 (49.0)	14 (14.5)		+38 (+16)
	β	2 (2.5)	50 (50.0)	15 (14.5)		
3-Deoxy-3-fluoro-D-glucose 6-phosphate	α	3 (3.9)	12 (12.0)	52 (53.0)	12 (12.0)	+268 (+458)
	β	<0.5 (<0.5)	13 (13.5)	51 (52.0)	13 (13.5)	
4-Deoxy-4-fluoro-D-glucose 6-phosphate	α			17 (15.0)	54 (49.0)	–119 (–189)
	β			16 (16.0)	47.5 (49.5)	

assay system for glucose phosphate isomerase has some advantages over previously published methods. It is not subject to the pitfalls that can arise from using a large number of coupling enzymes, and since it is a spectrophotometric assay it is more convenient than the pH-stat method (Dyson & Noltmann, 1965). The assay mixture consisted of glycylglycine (0.01M), Cresol Red (0.001%, w/v), MgCl_2 (0.015M), ATP (5mM) and phosphofructokinase (at least 2 units/ml). The decrease in E_{560} is a quantitative measure of the acid production in the reaction. All rates were measured at pH 8.9, by using a Cary model 16K.C kinetic spectrophotometer, the output of which was connected to a Varian strip-chart recorder, model G-2510. The cell compartment was thermostatically maintained at 30°C. The full-scale deflexion of the recorder was set to correspond to a ΔE_{560} of 0.02. Silica cells (1 cm light-path) were filled with all components of the reaction mixture except enzymes, in a total volume of 2.85 ml. Phosphofructokinase in 0.05M-cysteine (0.05 ml) was then added and the reaction was initiated by the addition of 0.1 ml of glucose phosphate isomerase (suitably diluted with water). Phosphofructokinase was dialysed with 0.05M-cysteine, pH 9.0, before dilution. The $\Delta E_{560}/\text{min}$ was proportional to the concentration of glucose phosphate isomerase in the range tested (0.1–1.0 unit/ml). When D-glucose 6-phosphate was the substrate a concentration of glucose phosphate isomerase of 0.1 unit/ml gave the required rate. All initial rates were determined in triplicate and the mean value was used for the determination of K_m and V_{max} values.

Data processing

Values of K_m and V_{max} in eqn. (1) were obtained by the method of Wilkinson (1961); see Bessell *et al.* (1972).

$$v = V_{\text{max}} \cdot [S] / (K_m + [S]) \quad (1)$$

Values of K_i , K_m and V_{max} in eqn. (2) for linear competitive inhibition were obtained by an analogous method by using a computer program written by Cleland (1963). The program was slightly modified

by Dr. L. I. Hart to run on the London University CDC 6600 computer.

$$v = V_{\text{max}} \cdot [S] / \{K_m(1 + [I]/K_i) + [S]\} \quad (2)$$

Results

Initial rates were obtained for both 3-deoxy-3-fluoro- and 4-deoxy-4-fluoro-D-glucose 6-phosphate by using the assay system described above. Each compound is therefore a substrate for glucose phosphate isomerase. In addition each of the products, 3-deoxy-3-fluoro- and 4-deoxy-4-fluoro-D-fructose 6-phosphate respectively, must be a substrate for the coupling enzyme phosphofructokinase. The initial rate for each compound was measured at three different concentrations (4, 8, and 12 units/ml) of phosphofructokinase. No difference in the rates at these three concentrations was observed, showing that the rate being followed was that of glucose phosphate isomerase. An equation derived by McClure (1969) for coupled enzyme assays enables calculation of the amount of auxiliary enzyme needed to attain a steady state after a short lag time (6s). Assuming a K_m value of 0.05 mM for D-fructose 6-phosphate for phosphofructokinase (rabbit muscle) (Kee & Griffin, 1972) the amount of phosphofructokinase needed when D-glucose 6-phosphate is the substrate in the coupled assay is 2–3 units/ml. Since a similar amount of phosphofructokinase was needed when the deoxy-fluoro-D-glucose 6-phosphates were substrates, the K_m and V_{max} values for both 3-deoxy-3-fluoro- and 4-deoxy-4-fluoro-D-fructose 6-phosphate for phosphofructokinase must be of the same order as the values for D-fructose 6-phosphate. The kinetic constants for D-glucose 6-phosphate, 3-deoxy-3-fluoro-D-glucose 6-phosphate and 4-deoxy-4-fluoro-D-glucose 6-phosphate for yeast glucose phosphate isomerase are given in Table 2. The K_m value for D-glucose 6-phosphate of 0.87 mM differs from the value of 0.3 mM quoted by Salas *et al.* (1965).

2-Deoxy-2-fluoro-D-glucose 6-phosphate, 2-chloro-2-deoxy-D-glucose 6-phosphate, 2-deoxy-D-arabinohexose 6-phosphate and D-glucosamine 6-phosphate were all competitive inhibitors for glucose phosphate isomerase, and the K_i values are given in Table 3.

Table 2. Kinetic constants for substrates for glucose phosphate isomerase

For experimental details see the text. Values are means $\pm$ S.E.M.

Compound	K_m (mM)	Relative V_{max}
D-Glucose 6-phosphate	0.87 ± 0.22	1
3-Deoxy-3-fluoro-D-glucose 6-phosphate	6.3 ± 1.3	0.007*
4-Deoxy-4-fluoro-D-glucose 6-phosphate	7.4 ± 1.3	0.33

* This low rate was not due to an impurity because a linear initial rate was observed for at least 5 min.

Table 3. K_i values obtained from the competitive inhibition by C-2-substituted D-glucose 6-phosphates of the isomerization of D-glucose 6-phosphate by yeast glucose phosphate isomerase

For experimental details see the text. Values are means $\pm$ S.E.M.

Compound	K_i (mM)	K_m (mM) for D-glucose 6-phosphate
2-Chloro-2-deoxy-D-glucose 6-phosphate	0.27 ± 0.02	1.03 ± 0.08
2-Deoxy-2-fluoro-D-glucose 6-phosphate	11.3 ± 2.5	0.85 ± 0.08
2-Deoxy-D-arabino-hexose 6-phosphate	1.9 ± 0.2	0.86 ± 0.07
D-Glucosamine 6-phosphate	2.9 ± 0.9	0.81 ± 0.08

Fig. 1. Competitive inhibition of yeast glucose phosphate isomerase by 2-chloro-2-deoxy-D-glucose 6-phosphate

The variation of the reciprocal of the initial rate is plotted against the reciprocal of the D-glucose 6-phosphate concentration for several constant concentrations of 2-chloro-2-deoxy-D-glucose 6-phosphate: □, 4.9 mM; ○, 0.98 mM; △, none. For experimental details see the text.

The competitive inhibition of the D-glucose 6-phosphate reaction by 2-chloro-2-deoxy-D-glucose 6-phosphate is shown by a double-reciprocal plot in Fig. 1.

Discussion

^1H and ^{19}F n.m.r. studies on the deoxyfluoro-D-glucoses (Phillips & Wray, 1971; Barford *et al.*, 1971)

have shown that these sugars, like D-glucose (Angyal, 1969), exist almost entirely (>98%) in aqueous solution as a mixture of the two pyranose forms, each pyranose form assuming a C1 conformation.

Since the ^1H - ^{19}F coupling constants for the deoxyfluoro-D-glucose 6-phosphates (see Table 1) are very similar to those for the non-phosphorylated compounds the deoxyfluoro-D-glucose 6-phosphates probably also exist, in aqueous solution, in the C1 conformation of the pyranose form.

Thus, this series of deoxyfluoro-D-glucose 6-phosphates is suitable for a study of the specificity of enzymes utilizing D-glucose 6-phosphate as a substrate, since the replacement of OH by F has little effect on the conformation of the sugars in aqueous solution.

Some of the possible effects of replacing a hydroxyl group by fluorine on the binding of a sugar to an enzyme (Bessell *et al.*, 1972) are, briefly, (a) the number of possibilities for hydrogen-bond interactions are decreased, since fluorine is only capable of accepting a hydrogen bond; (b) the inductive effect of a fluorine substituent will increase the acidity of vicinal hydroxyl groups [i.e. in the grouping $\text{CF}-\text{C}(\text{OH})$]; (c) the inductive effect can also alter the reaction rate if the fluorine substituent is sufficiently close to the part of the substrate involved in the reaction (Bessell & Thomas, 1973).

The facts that a large number of sugar phosphates, which are competitive inhibitors of glucose phosphate isomerase, are known, and also that phosphate itself is a competitive inhibitor (Dyson & Noltmann, 1968), suggest that the ionic binding of the phosphate moiety is of major importance in the binding of substrates and inhibitors.

Although the hydroxyl groups of the sugar are of minor importance in the binding, the D-glucose configuration of C-3, C-4 and C-5 is necessary to obtain a binding of the order of that of D-glucose 6-phosphate (Grazi *et al.*, 1960). The effect, therefore, of replacing a hydroxyl group of D-glucose 6-phosphate by a fluorine substituent would not be expected to be as large as in the binding of deoxyfluoro-D-glucoses to hexokinase (Bessell *et al.*, 1972), where a 500-fold decrease in binding was observed when HO-3 was replaced by a fluorine substituent. Nevertheless, the

10-fold decrease in binding that was observed when HO-3 and HO-4 of D-glucose 6-phosphate were replaced by a fluorine substituent might represent a comparable effect to that observed with the deoxy-fluoro-D-glucose (the K_m and K_i values given in Tables 2 and 3 are assumed to be of the same order as the K_i values). The role of HO-3 and HO-4 on the active site cannot be solely as a hydrogen-bond acceptor.

The low relative V_{max} value (0.007) of the 3-fluoro derivatives is noteworthy, since that of the 4-fluoro derivative is 0.33. This dramatic effect on the reaction rate is not explained by the inductive effect of the fluorine substituent, since this should facilitate rather than hinder the formation of the proposed enediol intermediate. The C-2 proton should be easier to abstract by the basic imidazole nitrogen atom (see the mechanism proposed by Dyson & Noltmann, 1968). Only if the mechanism were one involving hydride transfer from the substrate to the enzyme, with D-arabino-2-hexosulose 6-phosphate (D-glucose 6-phosphate) as an intermediate, would the inductive effect of fluorine be an explanation of the low relative V_{max} value. This mechanism was considered by Rose & O'Connell (1961) to be less likely than the mechanism they proposed.

It would seem therefore that although HO-3 and HO-4 have similar roles in the binding of the substrate to the enzyme, the role of HO-3 in the isomerization is more important. For a fast stereospecific isomerization to occur, the *cis* enediol grouping (Rose & O'Connell, 1961) must be fairly rigid in the active site. Rigidity of conformation would presumably be lost if the hydrogen-bond donation of HO-3 was destroyed. This effect would not be so pronounced with HO-4.

The C-2 substituted sugars are of interest as inhibitors, since they could form a substituted enediol structure but cannot be converted into product. The electron-withdrawing substituents (F, Cl and NH_3^+) should facilitate the removal of the proton from C-2, and hence 2-deoxy-2-fluoro-D-glucose 6-phosphate, 2-chloro-2-deoxy-D-glucose 6-phosphate and D-glucosamine 6-phosphate might be expected to be good inhibitors. The K_i values for these compounds are not consistent with this idea, since the 2-chloro derivative is a far better inhibitor than the 2-fluoro derivative, whereas fluorine is more strongly electron-withdrawing than is chlorine.

Although the size of a chlorine substituent is similar to that of a hydroxyl group there appears to be no correlation between inhibitor binding and the size of substituent. Steric factors, therefore, may not be important as far as C-2 is concerned.

Since sugar phosphates, which can only exist in an acyclic form, are the best competitive inhibitors of glucose phosphate isomerase, the ease with which a cyclic sugar phosphate can ring open into the acyclic

form may well determine its ability to bind to the enzyme. A substituent at C-2 will affect the rate of ring opening. The rates of mutarotation of these C-2-substituted compounds would be an indication of the rate of ring opening, but these could not be determined, because the D-glucose 6-phosphate derivatives were obtained as equilibrium mixtures. The rates of mutarotation are also not known for the corresponding halogen derivatives of D-glucose, since they crystallize as mixtures of anomers with a ratio similar to the equilibrium value in water (Adamson & Foster, 1969; Adamson *et al.*, 1970). The fact that 2-chloro-2-deoxy-D-glucose 6-phosphate has a lower K_i value than the K_m value of D-glucose 6-phosphate for glucose phosphate isomerase is consistent with the view that HO-2 does not play an important role in the binding. Since 2-chloro-2-deoxy-D-glucose is a substrate for yeast hexokinase (Bessell *et al.*, 1972) and 2-chloro-2-deoxy-D-glucose 6-phosphate is an inhibitor of glucose phosphate isomerase, 2-chloro-2-deoxy-D-glucose should be an inhibitor of glycolysis in yeast.

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