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The stereochemical course of yeast hexokinase-catalysed phosphoryl transfer by using adenosine 5′-[γ(*S*)-^{16}O,^{17}O,^{18}O]triphosphate as substrate

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Adenosine 5′-[γ(*S*)-^{16}O, ^{17}O, ^{18}O]triphosphate has been synthesized and used to determine the stereochemical course of phosphoryl transfer catalysed by yeast hexokinase. The chirality at phosphorus of the D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate formed was analysed, after cyclization and methylation, by ^{31}P n.m.r. spectroscopy. The phosphoryl transfer was found to occur with inversion of configuration, with a stereoselectivity in excess of 94%. The simplest interpretation of this result is that the phosphoryl group is transferred between substrates in the enzyme–substrate ternary complex by an 'in line' mechanism.

It is now generally agreed that yeast hexokinase follows a random sequential kinetic mechanism, with the addition of glucose before ATP and the release of glucose 6-phosphate after ADP being the preferred pathways (Colowick, 1973; Purich *et al.*, 1973). Kinetic methods, however, are unable to distinguish between the various ways in which phosphoryl transfer within the ternary complexes may take place. It is primarily for this reason that considerable interest has recently been shown in methods for investigating the stereochemical course of enzyme-catalysed phosphoryl-transfer reactions (Eckstein, 1975, 1979; Knowles, 1980).

Four mechanisms are possible for the transfer of the phosphoryl group in the interconversion of the ternary complexes of a phosphokinase, namely (i) the associative 'in line' mechanism, (ii) the dissociative mechanism, (iii) the adjacent mechanism, with pseudorotation (or 'turnstile' rotation) and (iv) the double-displacement mechanism (Lowe & Sproat, 1978). The first mechanism will occur with inversion of configuration at phosphorus, and it seems likely that the second mechanism will also occur with inversion of configuration since the enzyme must bind the metaphosphate intermediate tightly to prevent it being captured by solvent water, although the possibility of partial (or total) racemization cannot be excluded. The third mechanism should lead to retention of configuration, as should the fourth mechanism, if the reasonable assumption is made that the two phosphoryl-transfer steps follow the same stereochemical course. A stereochemical analysis of phosphoryl transfer should therefore eliminate at least two of the four proposed mechanisms.

The stereochemical course of a substantial number of phosphokinases has been investigated by using chiral [^{18}O]phosphorothioates, and it has been tacitly assumed that they will follow the same stereochemical course as that of the natural substrate (Eckstein, 1975, 1979; Knowles, 1980). This assumption has, however, only been tested with glycerol kinase, where it was found that both thiophosphoryl and phosphoryl transfer occur with inversion of configuration at phosphorus (Pliura *et al.*, 1980; Blättler & Knowles, 1979). However, it is not justified to assume that thiophosphate analogues (which are invariably poorer substrates than the natural phosphate esters) will always follow the same stereochemical course as the natural substrate.

We have recently developed a general route for the synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters of known absolute configuration (Cullis & Lowe, 1978, 1981), and have also developed an analytical method based on ^{31}P n.m.r. spectroscopy for determining the chirality at phosphorus of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate (Jarvest *et al.*, 1981). If, therefore, the stereochemical course of phosphoryl transfer catalysed by hexokinase can be rigorously established, a method for the analysis of any phosphokinase will be available, since ATP is a common substrate for all these enzymes.

Hexokinase catalyses the thiophosphorylation of glucose with the same stereochemical course as glycerol kinase (Orr *et al.*, 1978), but at a rate that must be exceedingly slow (Schlimme *et al.*, 1973; Gratecos & Fischer, 1974). It has also been shown that glycerol kinase catalyses both the thiophosphorylation and the phosphorylation of glycerol

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with inversion of configuration (Pliura *et al.*, 1980; Blättler & Knowles, 1979). It was therefore concluded, but not proved, that hexokinase also catalyses the phosphorylation of glucose with inversion of configuration at phosphorus (Blättler & Knowles, 1979). In view of the importance of this enzyme to our subsequent analysis of the stereochemical course of other phosphokinases, it was considered essential to determine directly the stereochemical course of yeast-hexokinase-catalysed phosphoryl transfer by using adenosine 5'-[$\gamma(S)$-^{16}O,^{17}O,^{18}O]triphosphate as substrate.

Materials and methods

Yeast hexokinase (type C-301) was obtained from Sigma (London) Chemical Co., as a crystalline suspension in 3.2M-$(NH_4)_2SO_4$ with an activity of 400units/mg of protein. It was shown by gel electrophoresis to be a mixture of the A and B isoenzymes, the former predominating. We thank Professor E. A. Barnard (Department of Biochemistry, Imperial College, London S.W.7, U.K.) for arranging for this to be determined in his laboratory. Glucose 6-phosphate dehydrogenase and pyruvate kinase were also obtained from Sigma (London) Chemical Co.

(1*R*,2*S*)-1,2-[1-^{18}O]Dihydroxy-1,2-diphenylethane was prepared as previously described (Cullis & Lowe, 1978, 1981) from (2*S*)-[1-^{18}O]benzoin, which was 92% (*S*) and 8% (*R*), was labelled with 97 atom % ^{18}O and was contaminated with 6% (1*S*,2*S*)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane. $P^{17}OCl_3$ was prepared from PCl_5 and $H_2{}^{17}O$ as previously described (Cullis & Lowe, 1978, 1981), and, on conversion of a portion into isotopically labelled trimethyl phosphate with methanol, had an isotope content of 3.3 atom % ^{16}O, 43.5 atom % ^{17}O and 53.2 atom % ^{18}O.

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 121.5MHz, except for routine spectra, which were recorded on a Bruker WH 90 FT spectrometer at 36.43MHz. All spectra are proton noise-decoupled, except where otherwise indicated, and the chemical shifts (δ_P) are measured with reference to external trimethyl phosphate; signals downfield from the reference are assigned positive chemical shifts. pH measurements were made on a Radiometer pH meter, standardized with BDH buffers.

Trans-2-(adenosine 5'-diphospho)-4,5-diphenyl-1,3,2-dioxaphospholan-2-one

This bis-sodium salt of adenosine 5'-diphosphate (471mg, 1mmol) was treated with Dowex 50W (H^+ form) resin in aqueous solution. After filtration and evaporation of the filtrate under reduced pressure, followed by the addition and evaporation of several volumes of dry methanol, methanol (25ml) and tri-n-octylamine (706mg, 2mmol) were added to the free acid and the mixture was vigorously shaken until the nucleotide dissolved. Evaporation of solvent under reduced pressure followed by evaporation of dry pyridine (4 × 50ml) gave adenosine 5'-diphosphate bis-(tri-n-octylammonium) salt.

Trans-2-chloro-4,5-diphenyl-1,3,2-dioxaphospholan-2-one was prepared *in situ* by treatment of *meso*-hydrobenzoin (214mg, 1mmol) with $POCl_3$ (94μl, 1mmol) in dry pyridine (Ukita *et al.*, 1961; Ukita, 1961; Cullis & Lowe, 1978, 1981). To this solution was added, with exclusion of moisture, tri-n-octylamine (1.06g, 3mmol), which had been dried by co-evaporation of dry pyridine. To this stirred solution at 0°C was added over 5min a solution of the bis-(tri-n-octylammonium) salt of adenosine 5'-diphosphate in dry pyridine (2ml) and the mixture was stirred for a further 5min, after which the pyridine was rapidly removed under reduced pressure and the last traces of solvent were removed by co-evaporation with dry dimethylformamide to give *trans*-2-(adenosine 5'-diphospho)-4,5-diphenyl-1,3,2-dioxaphospholan-2-one, δ_P (p.p.m.) (pyridine) −0.65(d, $^2J_{P_\gamma P_\beta}$ 22.8Hz, P_γ; undecoupled, double triplet, $^3J_{P_\gamma H}$ 7.4Hz), −15.24 (d, $^2J_{P_\alpha P_\beta}$ 19.9Hz, P_α; undecoupled-broad d) and −26.5 (t, $^2J_{P_\alpha P_\beta} = {}^2J_{P_\beta P_\gamma}$ = 21.4Hz, P_β; undecoupled-unchanged).

Adenosine 5'-triphosphate

A solution of the *trans*-2-(adenosine 5'-diphospho)-4,5-diphenyl-1,3,2-dioxaphospholan-2-one in dry dimethylformamide (25ml) containing dry tri-n-octylamine (353mg, 1mmol) was hydrogenolysed over 10% Pd/charcoal (0.6g) that had been dried in a vacuum desiccator over P_2O_5. The reaction was complete (approx. 60ml of H_2 absorbed) in 1.5h. The mixture was filtered through a glass-fibre filter paper, supported on a sinter funnel, and the residue was washed with dimethylformamide and then 300mM-NH_3 in aq. ethanol (1:1, v/v; 500ml). The filtrate and washings were concentrated under reduced pressure, partitioned between diethyl ether and water to remove diphenylethane and tri-n-octylamine, and the aqueous layer was evaporated under reduced pressure to give the tetrakis-ammonium salt of ATP. It was dissolved in triethylammonium hydrogen carbonate buffer (pH8.5, 200mM), applied to a column of DEAE-Sephadex A-25 (approx. 100ml) and eluted with a gradient of triethylammonium hydrogen carbonate buffer (100–600mM) over 24h at a flow rate of 82ml/h; 15min fractions were collected. Fractions containing ATP were identified by their absorbance

at 260nm; the ATP was shown to be a substrate for pyruvate kinase (Bücher & Pfleiderer, 1955). Removal of the solvent and buffer under reduced pressure gave adenosine 5'-triphosphate tetrakis-(triethylammonium) salt in an overall yield of 22%, δ_P (p.p.m.) (^2H_2O, pH9.0) −8.73 (d, $^2J_{P_\gamma P_\beta}$ 19.1 Hz, P_γ), −13.94(d, $^2J_{P_\alpha P_\beta}$ 19.9 Hz, P_α) and −24.62 (t, $^2J_{P_\beta P_\gamma} = {}^2J_{P_\alpha P_\beta} = 19.5$ Hz, P_β).

(2R,4S,5R)-2-(Adenosine 5'-diphospho)-4,5-diphenyl-1,3,2-[1-^{18}O]dioxaphospholan-2-[^{17}O]one

This was prepared in the same way as the unlabelled compound using (1*R*,2*S*)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane and $P^{17}OCl_3$ to prepare the isotopically labelled phosphorylating agent (Cullis & Lowe, 1978, 1981).

Adenosine 5'-[γ(S)-^{16}O,^{17}O,^{18}O]triphosphate

This was prepared by hydrogenolysis of (2*R*,4*S*,5*R*)-2-(adenosine 5'-diphospho)-4,5-diphenyl-1,3,2-[1-^{18}O]dioxaphospholan-2-[^{17}O]one, followed by purification as described for unlabelled ATP.

Yeast-hexokinase-catalysed phosphoryl transfer form ATP to D-*glucose*

To a solution containing the tetrakis-(triethylammonium) salt of ATP (20mM), $MgCl_2$ (6.6mM) and D-glucose (100mM) in triethanolamine buffer (pH8.0, 0.05M, 10ml) at room temperature was added a solution of yeast hexokinase (100μl, 400units/ml). The solution was kept at room temperature for 1.5h and then shaken with chloroform to denature the enzyme. The aqueous layer was degassed, diluted to the ionic strength of the starting buffer and applied to a DEAE Sephadex A-25 column (100ml) in 25mM-triethylammonium hydrogen carbonate buffer, pH8.0. The column was eluted with a gradient of triethylammonium hydrogen carbonate buffer (pH8.0, 25–400mM) over 24h at a flow rate of 82ml/h; 15min fractions were collected. Cl^- was detected by means of $AgNO_3$ solution and the fractions between Cl^- and ADP (detected by absorbance at 260nm) were examined enzymically with glucose 6-phosphate dehydrogenase/$NADP^+$ to identify those containing D-glucose 6-phosphate (Noltmann *et al.*, 1961). The fractions containing D-glucose 6-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-phosphate bis-(triethylammonium) salt (76mg, 0.16mmol), δ_P (p.p.m.) (20% ^2H_2O in water) + 1.82(s). This was treated with Dowex 50W (H^+ form) resin in aqueous solution, filtered and the filtrate was evaporated under reduced pressure to give D-glucose 6-phosphate. Dry dioxan was added followed by a solution of tri-n-octylamine (57mg, 0.16mmol) in dry dioxan. Methanol was added to assist formation of the salt. The solvent was removed and the D-glucose 6-phosphate mono-(tri-n-octylammonium) salt formed was thoroughly dried by addition and evaporation of several volumes of dry dioxan.

Yeast-hexokinase-catalysed phosphoryl transfer from adenosine 5'-[γ(S)-^{16}O,^{17}O,^{18}O[triphosphate to D-*glucose*

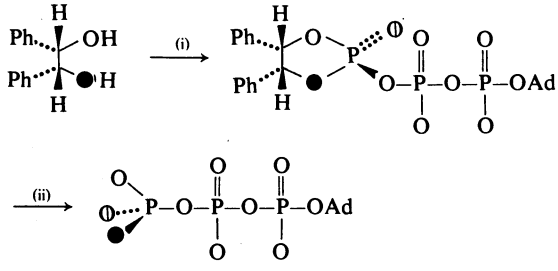
This was accomplished as described for the unlabelled ATP and the D-glucose 6-[^{16}O, ^{17}O,^{18}O]-phosphate isolated as before.

Analysis of the chirality at phosphorus of D-*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

Adenosine 5'-[γ(*S*)-^{16}O,^{17}O,^{18}O]triphosphate was prepared by the general method of synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate esters (Cullis & Lowe, 1978, 1981) as outlined in Scheme 1. In a preliminary investigation of the synthetic route using unlabelled *meso*-hydrobenzoin and $POCl_3$, it was shown by ^{31}P n.m.r. spectroscopy that ADP as its bis-(tri-n-octylammonium) salt is exclusively phosphorylated at the β-phosphate residue, to give the *trans*-diastereoisomer (Cullis & Lowe, 1981). After hydrogenolysis, the ATP was isolated and shown to be an effective substrate for pyruvate kinase. The



(i) (*a*) P⊖ Cl_3, pyridine

(*b*) 3 equiv. of tri-n-octylamine

(*c*) Bis-(tri-n-octylammonium) salt of ADP

(ii) H_2, Pd/C, dimethylformamide, tri-n-octylamine

Scheme 1. *The route for the synthesis of adenosine 5'-[γ(S)-^{16}O,^{17}O,^{18}O]triphosphate*

⊖ = ^{17}O; ● = ^{18}O.

synthesis was then performed with (1*R*,2*S*)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane and $P^{17}OCl_3$ (Scheme 1) (Cullis & Lowe, 1978, 1981). The absolute configuration at phosphorus of the adenosine 5′-[γ(*S*)-^{16}O,^{17}O,^{18}O]triphosphate follows from the known absolute configuration of the isotopically labelled diol and the *trans*-stereochemistry of the isotopically labelled 1,3,2-dioxaphospholan intermediate. The ^{31}P n.m.r. spectrum is shown in Fig. 1. The P_α and P_β resonances are the normal doublet and triplet respectively, but the P_γ resonance, which is a doublet in ATP, is seen on expansion (inset, Fig. 1) to consist of three doublets corresponding to ATP, [γ-^{18}O]ATP and [γ-$^{18}O_2$]ATP. The species containing ^{17}O directly bonded to ^{31}P are not observed in the ^{31}P n.m.r. spectrum owing to the nuclear electric quadrupole relaxation caused by the ^{17}O nucleus (Lowe *et al.*, 1979; Tsai, 1979; Tsai *et al.*, 1980). By subtracting the sum of all the P_γ resonances in this system from that expected for unlabelled ATP based on the P_β intensity, the proportion of adenosine 5′-[γ(*S*)-^{16}O,^{17}O,^{18}O]triphosphate can be calculated. The complete composition of the isotopically labelled ATP sample, taking into consideration the chiral purity of the [^{18}O]diol precursor, is shown in Table 1.

Adenosine 5′-[γ(*S*)-^{16}O,^{17}O,^{18}O]triphosphate was incubated with hexokinase and D-glucose at pH 8.0 for 1.5 h, by which time the reaction was complete. The D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate formed was cyclized and methylated to give the isotopically labelled axial and equatorial methyl D-glucose 4:6-cyclic monophosphate triesters as previously described (Jarvest *et al.*, 1981). The ^{31}P n.m.r.

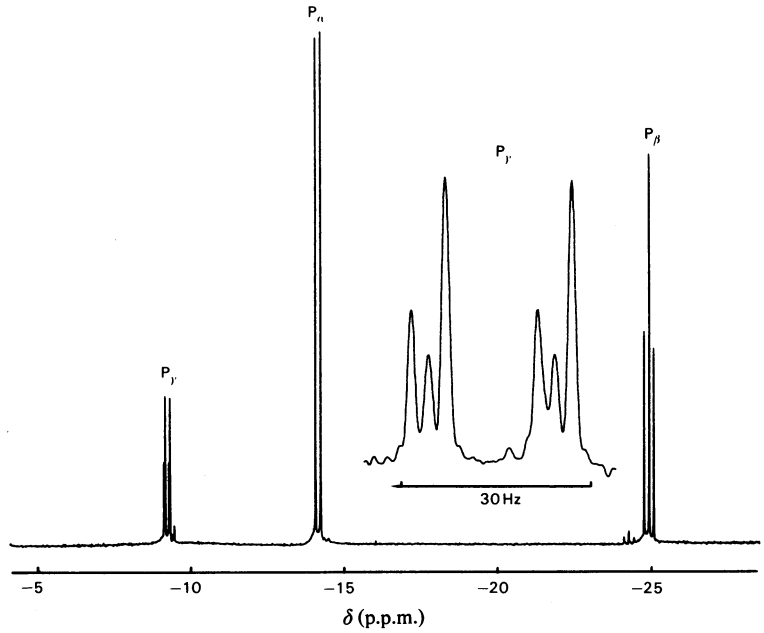


Fig. 1. *The ^{31}P n.m.r. spectrum (121.5 MHz) of adenosine 5′-[γ(S)-^{16}O,^{17}O,^{18}O]triphosphate (and its isotopomers) in 100 mM 2-amino-2-methylpropane-1,3-diol hydrochloride, pH 9.0, containing 10 mM-EDTA and 25% ^2H_2O*
The inset is the expanded P_γ resonance. The ^{31}P n.m.r. parameters are: sweep-width, 3000 Hz; pulse width 15 μs (angle, 70°); acquisition time, 1.36 s; memory size, 8 K; broad-band proton decoupling, Gaussian multiplication (line broadening −1.2 Hz, Gaussian broadening 0.3) in 8 K and Fourier transform in 32 K.

Table 1. *The composition of the isotopically labelled ATP sample determined from the ^{31}P n.m.r. spectrum (Fig. 1)*
From the known chiral purity of the [^{18}O]diol used in the synthesis of the [^{16}O,^{17}O,^{18}O]ATP, 87% will have the (*S*)- and 13% the (*R*)-configuration.

	Content (% of total)			
ATP species ...	ATP	[^{18}O]ATP	[$^{18}O_2$]ATP	[^{16}O,^{17}O,^{18}O]ATP
	20	15	37	28

spectrum is shown in Fig. 2, the assignments being shown on the spectrum. It has been shown that D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate is cyclized with inversion of configuration at phosphorus (Jarvest *et al.*, 1981). Since the relative intensities of the [$^{16}O_{ax.}$,$^{18}O_{eq.}$]- and [$^{16}O_{eq.}$,$^{18}O_{ax.}$]-triesters are reversed from those found in the analysis of D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate, the D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate obtained by the hexokinase-catalysed reaction must have the (*R*)-configuration at phosphorus. Hence phosphoryl transfer has occurred with inversion of configuration. From the data in Table 1 giving the actual composition of the isotopically labelled ATP, the

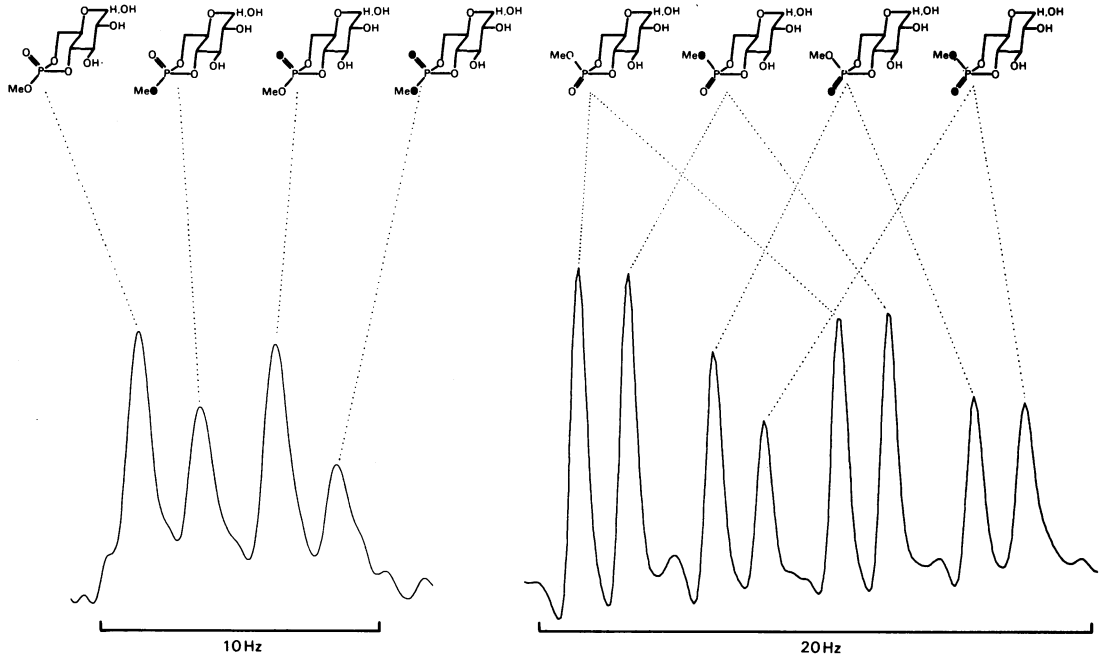


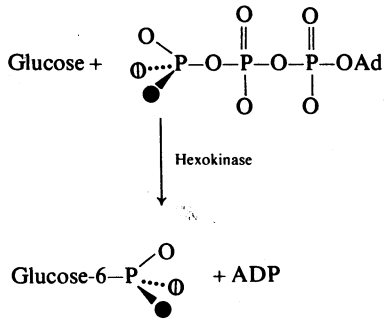
Fig. 2. *The ^{31}P n.m.r. spectrum (121.5 MHz) in [^{2}H]methanol/methanol/dimethyl sulphoxide (1:1:1, by vol.) of the equatorial and axial triesters derived by cyclization followed by methylation of* D-*glucose 6-[^{16}O,^{17}O,^{18}O]phosphate (and its isotopomers) obtained by the hexokinase-catalysed transfer of the γ-phosphoryl group from adenosine 5′-[γ(S)-^{16}O,^{17}O,^{18}O]triphosphate (and its isotopomers) to* D-*glucose*

The ^{31}P n.m.r. parameters are: sweep-width, 2000 Hz; pulse width, 16 μs (angle, 70°); acquisition time, 2.05 s; memory size, 8 K; broad-band proton decoupling, Gaussian multiplication (line broadening −1.0 Hz, Gaussian broadening 0.3) in 8 K and Fourier transform in 32 K. The vertical gain control for the equatorial triester is twice that used for the axial triester. The solvent mixture was chosen so that the anomers of the equatorial triester are not resolved, thereby improving the signal/noise ratio of these resonances. ● = ^{18}O.

Table 2. *Comparison of the observed relative peak intensities for the ^{31}P resonances in Fig. 2 with the calculated values expected for hexokinase-catalysed phosphoryl transfer with retention and inversion of configuration*

The isotopically labelled diastereoisomeric triesters are derived by cyclization followed by methylation of the D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate and its isotopomers; for the axial triesters, the average of both anomers is given except for the axial [$^{18}O_2$]triester where only the low-field anomer peak was used. The calculated values are those expected from the known isotopic composition of the ATP (Table 1) and the known chiral purity of the [^{18}O]diol used in the synthesis. ● = ^{18}O

	Peak intensity					
	Equatorial triester			Axial triester		
		Calculated			Calculated	
	Observed	Retention	Inversion	Observed	Retention	Inversion
MeO–P=O	1.04	0.99	0.99	0.99	0.99	0.99
Me●–P=O	0.75	1.00	0.73	1.00	0.73	1.00
MeO–P=●	1.00	0.73	1.00	0.73	1.00	0.73
Me●–P=●	0.56	0.49	0.49	0.54	0.49	0.49



Scheme 2. *The stereochemical course of hexokinase-catalysed [^{16}O,^{17}O,^{18}O]phosphoryl transfer with inversion of configuration at phosphorus*

⦶ = ^{17}O; ● = ^{18}O.

expected ratios of the isotopically labelled D-glucose 4,6-phosphate triesters can be calculated. The calculated values for phosphoryl transfer occurring with inversion and retention of configuration at phosphorus are compared in Table 2 with the experimental values derived from the ^{31}P n.m.r. spectrum (Fig. 2). From the ratio of the [$^{16}O_{ax.}$,$^{18}O_{eq.}$]- to [$^{16}O_{eq.}$,$^{18}O_{ax.}$]-equatorial and axial triesters it follows that hexokinase catalyses phosphoryl transfer from ATP to D-glucose with inversion of configuration and with a stereoselectivity in excess of 94% (Scheme 2).

Discussion

Hexokinase catalyses the transfer of the γ-phosphoryl group of adenosine 5′-[$\gamma(S)$-^{16}O,^{17}O,^{18}O]-triphosphate to D-glucose with inversion of configuration. This conclusion eliminates the adjacent mechanism followed by pseudorotation (or 'turnstile' rotation) and the double-displacement mechanism with a transient phosphoryl–enzyme intermediate. According to our original postulate, the mechanism must be either an associative or dissociative 'in line' mechanism, that is, the phosphoryl group must be transferred directly between substrates in the ternary complex, or by way of a metaphosphate intermediate. The latter mechanism, however, has been excluded by positional isotope-exchange experiments (Rose, 1980). Thus the simplest interpretation is that yeast hexokinase follows an associative 'in line' mechanism. One cannot, of course, rule out other mechanisms involving an odd number of 'in line' phosphoryl-transfer steps, but these would seem to be improbable. The observation that the Λ isomer of the β,γ-bidentate $Co(NH_3)_4ATP$ complex is a substrate for hexokinase, suggests that Mg^{2+} in the hexokinase–D-glucose–$MgATP^{2-}$ complex is bound to the β,γ-phosphate residues of ATP (Cornelius & Cleland, 1978), which is consistent with the associative mechanism.

All the phosphokinases that have been investigated by using chiral thio[^{18}O]phosphate analogues of the natural substrate have transferred the thiophosphoryl group with inversion of configuration (Eckstein, 1979; Knowles, 1980). The rate at which hexokinase catalyses the transfer of the thiophosphoryl group from adenosine 5′-[γ-^{18}O,γ-thio]triphosphate to D-glucose was not recorded (Orr *et al.*, 1978), but it had been reported previously that adenosine 5′-[γ-thio]triphosphate was not a substrate for hexokinase (Schlimme *et al.*, 1973; Gratecos & Fischer, 1974); presumably the transfer is exceedingly slow. The possibility that the mechanism followed by phosphoryl transfer was energetically unfavourable for thiophosphoryl transfer, allowing an alternative mechanistic pathway to become more favourable, had to be considered. We now know that with hexokinase, where thiophosphoryl transfer is very slow, both phosphoryl and thiophosphoryl transfer occur with inversion of configuration.

The X-ray structural analysis of the yeast hexokinase isoenzyme A–D-glucose complex has shown that the protein conformation is markedly different (Bennett & Steitz, 1978, 1980*a*,*b*) from the B isoenzyme, which crystallizes in the absence of D-glucose (Anderson *et al.*, 1978*a*). Evidence has been presented that this conformational change is induced on binding D-glucose, causing a 12° rotation of the two domains of the subunit, and is not due to the two isoenzymes having a fundamentally different folding of the polypeptide chain (McDonald *et al.*, 1979). In the hexokinase A–D-glucose complex the D-glucose is almost engulfed by the protein, only the 6-OH group being accessible, and even this is hydrogen bonded to Asp-195 (Anderson *et al.*, 1978*b*). The site to which $MgATP^{2-}$ binds has been deduced from binding studies with adenosine (Steitz *et al.*, 1977) and 8-bromoadenosine monophosphate (Shoham & Steitz, 1980). Model building of the triphosphate chain of ATP on to the adenosine in an extended conformation still leaves the γ-PO_3 group of ATP (the site is actually occupied by SO_4^{2-}) about 0.6 nm from the 6-OH group of D-glucose. The suggestion that a further conformational change occurs when $MgATP^{2-}$ binds to the hexokinase–D-glucose complex seems almost certainly correct in view of the evidence presented here for an 'in line' transfer of the phosphoryl group.

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