The stereochemical course of phosphoryl transfer catalysed by *Bacillus stearothermophilus* and rabbit skeletal-muscle phosphofructokinase with a chiral [^{16}O , ^{17}O , ^{18}O]phosphate ester

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Bacillus stearothermophilus and rabbit skeletal-muscle phosphofructokinases catalyse the transfer of the chiral [^{16}O , ^{17}O , ^{18}O]phosphoryl group from D-fructose 1[(*S*)- ^{16}O , ^{17}O , ^{18}O],6-bisphosphate to ADP with inversion of configuration at the phosphorus atom. D-Fructose 1[(*S*)- ^{16}O , ^{17}O , ^{18}O],6-bisphosphate was synthesized *in situ* from *sn*-glycerol 3[(*S*)- ^{16}O , ^{17}O , ^{18}O]phosphate. The simplest interpretation of these results is that the phosphoryl group is transferred between substrates in the enzyme substrate ternary complexes by an 'in-line' mechanism.

Phosphofructokinase (EC 2.7.1.11) catalyses the phosphorylation of D-fructose 6-phosphate by MgATP to give D-fructose 1,6-bisphosphate and MgADP, and is subject to regulation by a variety of intermediary metabolites (Bloxham & Lardy, 1973; Uyeda, 1979). The enzyme has been isolated from a number of sources, but the rabbit skeletal-muscle enzyme has been most extensively studied. Although kinetic studies are fraught with difficulty, mainly because the enzymic activity is influenced allosterically by its own substrates (Mg^{2+} , ATP and D-fructose 6-phosphate), there now seems no doubt that the enzyme follows a sequential pathway (Bloxham & Lardy, 1973; Bar-Tana & Cleland, 1974*a,b*; Uyeda, 1979).

Rabbit skeletal-muscle phosphofructokinase catalyses ATP–ADP exchange in the absence of D-fructose 6-phosphate, albeit at less than 1% of the overall forward reaction; this exchange is dependent on Mg^{2+} (Uyeda, 1970; Hulme & Tipton, 1971; Hanson *et al.*, 1973). Moreover, the enzyme catalyses the hydrolysis of ATP and D-fructose 1,6-bisphosphate at rates 0.06% and 0.4% of the overall forward and reverse reactions respectively (Uyeda, 1979). These results suggest the formation of a phosphoryl-enzyme intermediate, but it is quite uncertain as to whether substrate synergism (Bridger *et al.*, 1968) increases the rate of phosphoryl transfer to make the putative phosphoryl-enzyme a kinetically competent intermediate when both substrates and Mg^{2+} are present.

It was precisely this type of problem that led us to develop methodology for determining the stereochemical course of phosphoryl transfer reactions by

using chiral [^{16}O , ^{17}O , ^{18}O]phosphate esters (Cullis & Lowe, 1978, 1981; Cullis *et al.*, 1981; Jarvest *et al.*, 1981). Stereochemical analysis should make a clear distinction between the alternative possibilities, since, if the chiral [^{16}O , ^{17}O , ^{18}O]phosphoryl group is transferred directly between substrates in the ternary complex, inversion of configuration at the phosphorus atom would be expected, whereas, if a phosphoryl-enzyme intermediate is on the reaction pathway, the double displacement should lead to overall retention of configuration. We have investigated the stereochemical course of both the *Bacillus stearothermophilus* and the rabbit skeletal-muscle enzymes, since they differ substantially in molecular and allosteric properties; the *B. stearothermophilus* enzyme is being investigated crystallographically (Evans & Hudson, 1979; Evans *et al.*, 1981).

Materials and methods

Materials

Bacillus stearothermophilus phosphofructokinase (150 units/mg of protein; assayed as described by Boehringer, Mannheim, Germany) was a gift from Dr. P. R. Evans. 3-Chloroacetyl phosphate dimethyl ketal was a gift from Dr. S. G. Waley; it was converted into 3-chloroacetyl phosphate immediately before use (Hartman, 1970). 1,2-Dipalmitoyl-*sn*-glycerol (1,2-dipalmitin), rabbit skeletal-muscle phosphofructokinase (200 units/mg of protein; assayed as above), *sn*-glycerol 3-phosphate dehydrogenase (rabbit muscle), fructose bisphosphate aldolase (rabbit muscle), phospho-

glucose isomerase (yeast), D-glucose 6-phosphate dehydrogenase (baker's yeast) and L-lactate dehydrogenase were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), and were the highest grade available. $P^{17}OCl_3$ was prepared as previously described (Cullis & Lowe, 1981), and was shown by mass spectrometry after conversion into trimethyl phosphate to have the isotopic composition 3.3 atoms % ^{16}O , 43.5 atoms % ^{17}O and 53.2 atoms % ^{18}O .

Measurement of n.m.r. spectra

^{31}P n.m.r. chemical shifts are reported for unlabelled compounds with positive chemical shifts downfield from trimethyl phosphate, and were measured by using an external ^2H_2O field-frequency lock on a Bruker WH90 FT spectrometer at 36.43 MHz. The ^{18}O isotope shifts on the ^{31}P n.m.r. spectra were recorded on a Bruker WH300 FT spectrometer with quadrature detection at 121.5 MHz.

sn-Glycerol 3-[(*S*)- ^{16}O , ^{17}O , ^{18}O]phosphate

(1*R*,2*S*) - [1 - ^{18}O]1,2 - Dihydroxy - 1,2 - diphenylethane (216 mg, 1.0 mmol) (Lowe & Potter, 1981) in pyridine (3 ml) was added to a stirred solution of $P^{17}OCl_3$ (94 μ l, 1.0 mmol) in pyridine (0.5 ml) at 0°C over 2 h. The solution was stirred at room temperature for a further 1 h. 1,2-Dipalmitoyl-*sn*-glycerol (569 mg, 1.0 mmol) in pyridine (6 ml) was added to the solution at 0°C, and the solution was stirred overnight. The solvent was removed *in vacuo*, and the last traces of pyridine were removed by addition and evaporation of ethyl acetate (twice). The residue was treated with ethyl acetate (10 ml), and pyridine hydrochloride was removed by filtration. The product had $\delta_p + 12.2$ p.p.m. (in pyridine).

The ethyl acetate solution was added to 10% palladium on charcoal catalyst (300 mg, then dried in a vacuum desiccator over P_2O_5) in ethyl acetate (10 ml), and the mixture was stirred under H_2 for 2.5 h. The mixture was filtered, and the catalyst was washed with hot ethyl acetate followed by chloroform. The solvent was removed to leave the free acid ($\delta_p - 3.1$ p.p.m. in $CHCl_3$) and diphenylethane.

This material was dissolved in chloroform (120 ml) and methanol (20 ml), and methanolic 0.5 M-NaOH (40 ml) was added (cf. Hajra *et al.*, 1968). After 10 min the solution was diluted with chloroform/methanol (2:1, v/v) (20 ml) and extracted with water (50 ml). The organic layer was extracted with chloroform/methanol/water (3:47:48, by vol.). The combined aqueous extracts were adjusted to pH 8.0 with Dowex 50W resin (H^+ form), and the solvent was removed to give *sn*-glycerol 3[(*S*)- ^{16}O , ^{17}O , ^{18}O]phosphate disodium salt (0.6 mmol, 60% yield), $\delta_p + 1.6$ p.p.m. (in water).

From the high-field ^{31}P n.m.r. spectrum it was found that the species *sn*-glycerol 3-[(^{16}O)₃]phosphate, *sn*-glycerol 3-[(^{16}O , ^{18}O)]phosphate and *sn*-glycerol 3-[(^{16}O , ^{18}O)₂]phosphate were in the proportions 0.05:0.35:1.00.

B. stearothermophilus phosphofructokinase-catalysed [^{16}O , ^{17}O , ^{18}O]phosphoryl transfer reaction

sn-Glycerol 3-phosphate dehydrogenase (200 units), fructose biphosphate aldolase (500 units), phosphofructokinase (500 units), phosphoglucose isomerase (1250 units), D-glucose 6-phosphate dehydrogenase (1000 units) and L-lactate dehydrogenase (1800 units) were dissolved in 0.2 M-Tris/HCl buffer, pH 9.0 (2 ml), containing magnesium acetate (6 mM), KCl (4 mM) and 3-chloroacetol phosphate (2 mg/ml) and kept for 15 min. (All enzyme activity units are expressed as μ mol of substrate transformed/min.) To this solution was then added ADP (1.0 mmol, 20 mM), NAD^+ (0.1 mmol, 2 mM), $NADP^+$ (0.6 mmol, 12 mM), DL-glyceraldehyde 3-phosphate (1.0 mmol, 20 mM), sodium pyruvate (2.5 mmol, 50 mM), *sn*-glycerol 3-[(*S*)- ^{16}O , ^{17}O , ^{18}O]phosphate (0.5 mmol, 10 mM) and bovine serum albumin (50 mg) in 0.2 M-Tris/HCl buffer, pH 9.0 (48 ml), containing magnesium acetate (6 mM) and KCl (4 mM), and the solution was kept overnight at 37°C. The reaction was terminated by addition of EDTA (150 mg, 0.4 mmol) and vigorous agitation with chloroform. The chloroform layer was twice extracted with water, and the aqueous extracts were pooled. The solution was applied to a column (150 ml) of DEAE-Sephadex A-25 and eluted with a linear gradient of triethylammonium bicarbonate (200–600 mM) over 24 h at a flow rate of 110 ml/h, with collection of five fractions/h. ATP (0.15 mmol, yield 30%) was eluted in fractions 49–60 (430 mM buffer) and the buffer was removed. The 121.5 MHz ^{31}P n.m.r. spectrum showed three doublets for $P_{(p)}$ in the proportions [γ - ^{16}O]₃ATP (2.10), [γ - ^{16}O , ^{18}O]-ATP (0.40) and [γ - ^{16}O , ^{18}O]₂ATP (1.00). Comparison of the sum of the integration of all these $P_{(p)}$ resonances with that of $P_{(p)}$ allowed (by difference) the amount of [γ - ^{16}O , ^{17}O , ^{18}O]ATP to be calculated. From these data the approximate composition of the ATP at $P_{(p)}$ was calculated to be: [γ - ^{16}O]₃ATP, 48%; [γ - ^{16}O , ^{18}O]₂ATP, 10%; [γ - ^{16}O , ^{18}O]₂ATP, 23%; [γ - ^{16}O , ^{17}O , ^{18}O]ATP, 19%.

Rabbit muscle phosphofructokinase-catalysed [^{16}O , ^{17}O , ^{18}O]phosphoryl transfer reaction

This reaction was conducted in the same manner as for the *B. stearothermophilus* enzyme, except that rabbit skeletal-muscle enzyme (400 units) was used. It was performed on 0.3 mmol of *sn*-glycerol 3-[(*S*)- ^{16}O , ^{17}O , ^{18}O]phosphate, and the yield of ATP was 0.12 mmol (40%).

Analysis of the chirality at phosphorus of [γ - ^{16}O , ^{17}O , ^{18}O]ATP

The [γ - ^{16}O , ^{17}O , ^{18}O]ATP was incubated with D-glucose and yeast hexokinase to give D-glucose 6-[(^{16}O , ^{17}O , ^{18}O)phosphate]; this occurs with inversion of configuration (Lowe & Potter, 1981). The D-glucose 6-[(^{16}O , ^{17}O , ^{18}O)phosphate] was then cyclized and esterified as previously described (Jarvest *et al.*, 1981), and the 121.5 MHz ^{31}P n.m.r. spectrum was recorded.

Results and discussion

ATP is a common substrate for all phosphokinases. If phosphoryl transfer from D-fructose 1[(S)- ^{16}O , ^{17}O , ^{18}O], 6-bisphosphate to MgADP is effected by phosphofructokinase, the chirality at $\text{P}_{(\gamma)}$ in the [γ - ^{16}O , ^{17}O , ^{18}O]ATP could be established by

transferring the γ -phosphoryl group to D-glucose with yeast hexokinase, which occurs with inversion of configuration at the phosphorus atom (Lowe & Potter, 1981), and determining the chirality at the phosphorus atom of the D-glucose 6-[(^{16}O , ^{17}O , ^{18}O)phosphate] by ^{31}P n.m.r. spectroscopy (Jarvest *et al.*, 1981).

Phosphoryl transfer from D-fructose 1,6-bisphosphate to ADP to give D-fructose 6-phosphate and ATP is a thermodynamically unfavourable process. In order to achieve a satisfactory yield of ATP, the D-fructose 6-phosphate must be removed as it is formed. This was accomplished by having present, in the reaction medium, phosphoglucose isomerase and glucose 6-phosphate dehydrogenase with its co-enzyme, NADP $^{+}$. These enzymes convert D-fructose 6-phosphate into D-gluconolactone 6-phosphate, which spontaneously and irreversibly

Scheme 1. Enzymic route used for converting the phosphoryl group of *sn*-glycerol 3-[(*S*)- ^{16}O , ^{17}O , ^{18}O]phosphate into [γ - ^{16}O , ^{17}O , ^{18}O]ATP

The only phosphoryl transfer reaction is that catalysed by phosphofructokinase. $\oplus = ^{17}\text{O}$, $\bullet = ^{18}\text{O}$.

hydrolyses. In this way the synthesis of ATP by phosphofructokinase is made favourable.

Synthetic expediency led us to use *sn*-glycerol 3-[(*S*)- ^{16}O , ^{17}O , ^{18}O]phosphate as the source of D-fructose 1-[(*S*)- ^{16}O , ^{17}O , ^{18}O],6-bisphosphate. This conversion was accomplished *in situ* by having present in the reaction medium glycerophosphate dehydrogenase and NAD^+ (the coenzyme being regenerated by coupling to pyruvate and lactate dehydrogenase), the dihydroxyacetone [(*S*)-

^{16}O , ^{17}O , ^{18}O]phosphate so formed being converted into D-fructose 1[(*S*)- ^{16}O , ^{17}O , ^{18}O],6-bisphosphate by fructose bisphosphate aldolase. The complete enzymic system is shown in Scheme 1. The only phosphoryl transfer step involved is that catalysed by phosphofructokinase. It is important, however, that the concentration of D-glyceraldehyde 3-phosphate should not be excessively high, since this will inhibit fructose bisphosphate aldolase (Lai *et al.*, 1968). Moreover, it is important to ensure that triose

Scheme 2. Synthesis of *sn*-glycerol 3-[(*S*)- ^{16}O , ^{17}O , ^{18}O]phosphate

Reagents: (i) (a) $\text{P}^{17}\text{OCl}_3$, pyridine; (b) 1,2-dipalmitoyl-*sn*-glycerol, pyridine; (ii) (a) H_2 , Pd/C; (b) NaOH, methanol/ CHCl_3 . $\bigcirc = ^{17}\text{O}$, $\bullet = ^{18}\text{O}$.

Fig. 1. ^{31}P n.m.r. spectrum (121.5 MHz) of the equatorial and axial triesters derived by cyclization and subsequent methylation of D-glucose 6-[(*S*)- ^{16}O , ^{17}O , ^{18}O]phosphate obtained by yeast hexokinase-catalysed transfer to D-glucose of the γ -phosphoryl group from [γ - ^{16}O , ^{17}O , ^{18}O]ATP formed by *B. stearothermophilus* phosphofructokinase as outlined in Scheme 1

The solvent [33% (v/v) dimethyl sulphoxide in [$^2\text{H}_4$]methanol] was chosen so that the anomers of the equatorial triester were not separated. The ^{31}P n.m.r. parameters are: offset 2240 Hz, sweep width 2000 Hz, acquisition time 2.05 s, pulse width (angle) $16\ \mu\text{s}$ (75°), gaussian multiplication (line broadening $-1.0\ \text{Hz}$, gaussian broadening 0.35) in 8 K and Fourier transform in 32 K.

Table 1. Observed relative peak intensities of the ^{31}P n.m.r. resonances (from Fig. 1, average of both anomers of the axial triesters) of the ^{18}O -labelled diastereoisomeric triesters derived by cyclization followed by methylation of the D-glucose 6- ^{16}O , ^{17}O , ^{18}O]phosphate formed by yeast hexokinase from $[\gamma\text{-}^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\text{ATP}$ obtained as outlined in Scheme 1, compared with the calculated values expected for phosphoryl transfer by *B. stearothermophilus* phosphofructokinase with retention and inversion of configuration at the phosphorus atom

● = ^{18}O .

	Equatorial triester			Axial triester		
	Observed	Calculated		Observed	Calculated	
		Retention	Inversion		Retention	Inversion
$\text{MeO}-\text{P}=\text{O}$	3.32	3.52	3.52	3.45	3.52	3.52
$\text{Me}\bullet-\text{P}=\text{O}$	1.00	0.77	1.00	0.77	1.00	0.77
$\text{MeO}-\text{P}=\bullet$	0.71	1.00	0.77	1.00	0.77	1.00
$\text{Me}\bullet-\text{P}=\bullet$	0.30	0.47	0.47	0.51	0.47	0.47

phosphate isomerase is not present, since this enzyme interconverts dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate and would lead to the [(S)- ^{16}O , ^{17}O , ^{18}O]phosphoryl group appearing at both C-1 and C-6 in D-fructose 1,6-bisphosphate. To prevent this possibility all the enzymes were treated with 3-chloroacetol phosphate, a potent irreversible inhibitor of triose phosphate isomerase (Hartman, 1970), before the addition of substrates.

sn-Glycerol 3-[(S)- ^{16}O , ^{17}O , ^{18}O]phosphate was prepared by our general method of synthesis (Cullis & Lowe, 1981) from 1,2-dipalmitoyl-*sn*-glycerol as outlined in Scheme 2. It was incubated with the enzymes, coenzymes and substrates shown in Scheme 1. The $[\gamma\text{-}^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\text{ATP}$ was isolated, then incubated with D-glucose and yeast hexokinase, and the D-glucose 6- ^{16}O , ^{17}O , ^{18}O]phosphate was cyclized and esterified (Jarvest, Lowe & Potter, 1981). The ^{31}P n.m.r. spectrum of the product obtained with the *B. stearothermophilus* enzyme is shown in Fig. 1. The unexpectedly large resonances of the unlabelled triesters indicate that, in spite of the addition of 3-chloroacetol phosphate to the enzyme before the incubation with substrates, some isomerization of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate has occurred. Fortunately, this could be estimated from the isotopic composition of the labelled ATP by ^{31}P n.m.r. spectroscopy. By taking this into consideration, together with the known chiral purity of the [^{18}O]diol (Lowe & Potter, 1981), the isotopic composition of the $\text{P}^{17}\text{OCl}_3$ (3.3 atoms % ^{16}O , 43.5 atoms % ^{17}O and 53.2 atoms % ^{18}O) used in the synthesis, and the expected loss of isotope during the synthesis of *sn*-glycerol 3-[(S)- ^{16}O , ^{17}O , ^{18}O]phosphate and the cyclization of D-glucose 6- ^{16}O , ^{17}O , ^{18}O]phosphate (Jarvest *et al.*, 1981), it was possible to calculate the expected proportions of the isotopically labelled D-glucose 4,6-phosphate triesters. The stereochemical course of the reaction is determined by the ratio of the

$[\text{}^{16}\text{O}_{\text{ax.}}, \text{}^{18}\text{O}_{\text{eq.}}]\text{-}$ to $[\text{}^{18}\text{O}_{\text{ax.}}, \text{}^{16}\text{O}_{\text{eq.}}]\text{-}$ triesters. By comparing the observed relative intensities (from Fig. 1) with the calculated values for retention and inversion of configuration during the phosphofructokinase reaction (see Table 1), it is clear that *B. stearothermophilus* phosphofructokinase catalyses phosphoryl transfer with inversion of configuration at the phosphorus atom. The rabbit skeletal-muscle enzyme was also shown to catalyse phosphoryl transfer with inversion of configuration, the ratio of the $[\text{}^{18}\text{O}_{\text{ax.}}, \text{}^{16}\text{O}_{\text{eq.}}]\text{-}$ to $[\text{}^{16}\text{O}_{\text{ax.}}, \text{}^{18}\text{O}_{\text{eq.}}]\text{-}$ axial triesters being 0.80:1.00 compared with the calculated ratio of 0.77:1.00 for inversion of configuration (the intensity of the equatorial triesters was too small to measure accurately). The simplest interpretation of these results is that phosphoryl transfer occurs in the enzyme substrate ternary complexes by an 'in-line' mechanism.

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