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The stereochemical course of phosphoryl transfer catalysed by polynucleotide kinase (bacteriophage-T_4-infected *Escherichia coli* B)

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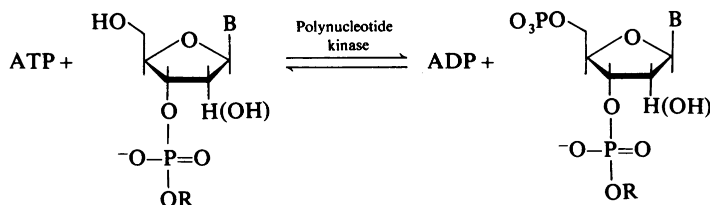
Polynucleotide kinase (bacteriophage-T_4-infected *Escherichia coli* B) catalyses the transfer of the [γ-^{16}O,^{17}O,^{18}O]phosphoryl group from 5′-[$\gamma(S)$-^{16}O,^{17}O,^{18}O]ATP to 3′-AMP with inversion of configuration at the phosphorus atom. The simplest interpretation of this observation is that the [γ-^{16}O,^{17}O,^{18}O]phosphoryl group is transferred directly from ATP to the co-substrate by an 'in-line' mechanism.

Polynucleotide kinase (bacteriophage-T_4-infected *Escherichia coli* B) (EC 2.7.1.78) catalyses the 5′-phosphorylation of nucleic acids, oligonucleotides and nucleoside 3′-phosphates (Scheme 1) (Richardson, 1965), and is currently enjoying widespread use in structural work with nucleic acids (Kleppe & Lillehaug, 1979). Thus, with [γ-^{32}P]-ATP, the enzyme catalyses ^{32}P-labelling of nucleic acids or oligonucleotides, which is an essential step in end-group analysis (Takanami, 1967; Richardson, 1971), 'fingerprinting' of oligonucleotides derived by nuclease digestion (Székely & Sanger, 1969; Southern, 1970; Murray, 1973) and nucleotide sequencing of nucleic acids (Maxam & Gilbert, 1977, 1980).

Moreover, the enzymic reaction is reversible, so that it is unnecessary to remove the 5′-phosphate group from the nucleic acid with phosphatase, since incubation with polynucleotide kinase, ADP and [γ-^{32}P]ATP will exchange the 5′-phosphate to give the 5′-[^{32}P]phosphate (van de Sande *et al.*, 1973). During this latter investigation it was found that the specificity for the ADP/ATP-binding site was low, and, in particular, when ATP and [^{32}P]nucleic acid were incubated with the enzyme, adenosine 5′-[δ-^{32}P]tetraphosphate was formed; the enzyme also showed some phosphatase activity. These observations were rationalized by suggesting that a phosphoryl-enzyme intermediate was involved (van de Sande *et al.*, 1973).

Initial-rate and product-inhibition studies showed that the enzyme follows a sequential pathway, i.e. both substrates bind to the enzyme to form a ternary complex before phosphoryl transfer takes place (Lillehaug & Kleppe, 1975), but whether the nucleic acid binds before or after the ATP appears to depend on its structure (Lillehaug *et al.*, 1976). The sequential mechanism is certainly consistent with the lack of polynucleotide kinase-catalysed ADP–ATP exchange, but substrate synergism (Bridger *et al.*, 1968) had been invoked in support of the putative phosphoryl-enzyme intermediate (van de Sande *et al.*, 1973).

In order to investigate whether phosphoryl transfer takes place directly between substrates in the enzyme ternary complex or by way of a phosphoryl-enzyme intermediate, the stereochemical course of polynucleotide kinase-catalysed phosphoryl transfer



Scheme 1. *Reaction catalysed by polynucleotide kinase*
R = H, nucleoside, oligonucleotide or polynucleotide; B = base.

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has been investigated with 5′-[$\gamma(S)$-$^{16}O,^{17}O,^{18}O$]-ATP. If a double-displacement mechanism with a phosphoryl-enzyme intermediate is involved retention of configuration would be expected, whereas if direct transfer between substrates occurs inversion of configuration should be observed.

We have developed a method based on ^{31}P n.m.r. spectroscopy for analysing the chirality at the phosphorus atom of [$^{16}O,^{17}O,^{18}O$]ATP (Jarvest *et al.*, 1980, 1981; Cullis *et al.*, 1981*a*). If 3′-AMP is used as the co-substrate and the 3′-phosphate subsequently removed with nuclease P_1, analysis of the chirality at the phosphorus atom of the residual 5′-[$^{16}O,^{17}O,^{18}O$]ATP should allow the stereochemical course of the phosphoryl transfer catalysed by polynucleotide kinase to be determined.

Materials and methods

Materials

Polynucleotide kinase (bacteriophage-T_4-infected *E. coli* B) was obtained from P–L Biochemicals (Milwaukee, WI, U.S.A.) [specific activity 30000 units/mg; 1 unit catalyses the transfer of 1 nmol of phosphate from ATP to polynucleotide in 30 min at 37°C by the method of Richardson (1971), except that yeast RNA was used as substrate] and nuclease P_1 (*Penicillium citrium*) (500 units/mg) from Sigma Chemical Co. (Poole, Dorset, U.K.).

5′-[$\gamma(S)$-$^{16}O,^{17}O,^{18}O$]ATP was prepared as previously described (Lowe & Potter, 1981). The isotopic composition of the $P^{17}OCl_3$ used in the synthesis was 3.3 atoms % ^{16}O, 43.5 atoms % ^{17}O and 53.2 atoms % ^{18}O, and the (1*R*,2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane (Cullis & Lowe, 1981) was 94% pure, with 6% of 1,2-dihydroxy-1,2-diphenylethane present. The ^{31}P n.m.r. spectrum showed the ratio of [γ-$^{16}O_2,^{18}O$]ATP to [γ-$^{16}O,^{18}O_2$]-ATP to be 0.18 : 1.00 and the ratio of the intensity of $P_{(\gamma)}$ to $P_{(\beta)}$ resonances to be 0.55 : 1.00 (Fig. 1). This indicates a 3% ring opening during hydrogenolysis, and gives a calculated isotopic composition of the 5′-[$\gamma(S)$-$^{16}O,^{17}O,^{18}O$]ATP of: [γ-$^{16}O_3$]ATP, 1%; [γ-$^{16}O_2,^{17}O$]ATP, 3%; [γ-$^{16}O_2,^{18}O$]ATP, 9%; [$\gamma(R)$-$^{16}O,^{17}O,^{18}O$]ATP, 1%; [$\gamma(S)$-$^{16}O,^{17}O,^{18}O$]-ATP, 38%; [γ-$^{16}O,^{18}O_2$]ATP, 48%.

Polynucleotide kinase-catalysed [$^{16}O,^{17}O,^{18}O$]phosphoryl transfer

To 10 ml of 100 mM-Tris/HCl buffer, pH 9.0, was added $MgCl_2$ (20.0 mg, 100 μmol, 10 mM), [$\gamma(S)$-$^{16}O,^{17}O,^{18}O$]ATP (100 μmol, 10 mM), 3′-AMP (55.0 mg, 150 μmol, 15 mM), dithiothreitol (7.5 mg, 50 μmol, 5 mM) and spermine (7.0 mg, 20 μmol, 2 mM), followed by polynucleotide kinase (325 units), and the solution was incubated at 37°C for 60 h. Nuclease P_1 (1.0 mg) was then added and the pH was adjusted to 7.5 with dilute HCl. After 1.5 h, the reaction was terminated by addition of EDTA (560 mg, 150 μmol) and vigorous agitation with chloroform. Ion-exchange chromatography was performed on a column of DEAE-Sephadex A-25 (20 ml) with a linear gradient of 50–200 mM-triethylammonium bicarbonate buffer, pH 8.0, run over 24 h at 50 ml/h, with collection of four fractions/h. 5′-[$^{16}O,^{17}O,^{18}O$]AMP was eluted in fractions 29–35

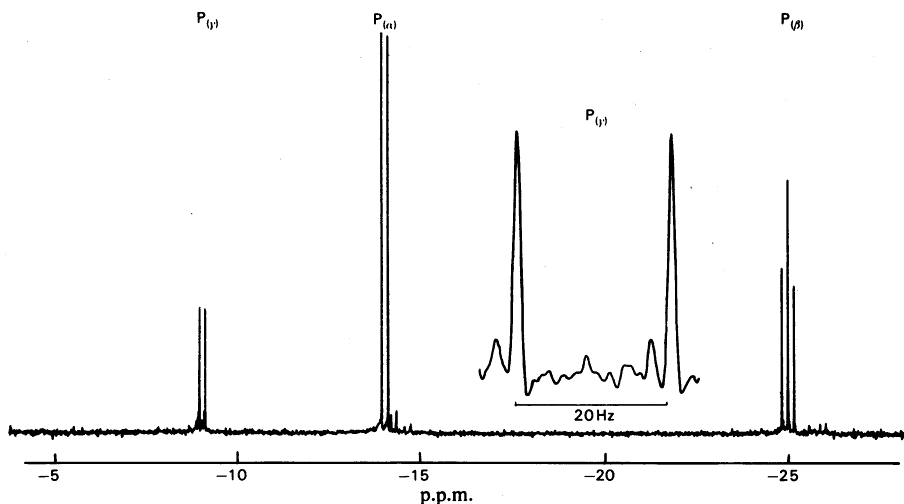


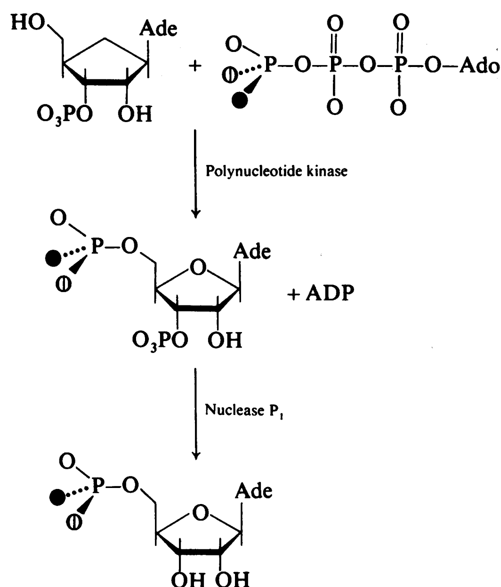
Fig. 1. *^{31}P n.m.r. spectrum (121.5 MHz) of 5′-[$\gamma(S)$-$^{16}O,^{17}O,^{18}O$]ATP in ^2H_2O (50%) containing EDTA at pH 9.0*
The ^{31}P n.m.r. parameters are: offset 900 Hz, sweep width 3000 Hz, acquisition time 1.36 s, pulse width (angle) 15 μs (75°), gaussian multiplication (line broadening −0.9 Hz, gaussian broadening 0.3) in 8 K and Fourier transform in 32 K. The inset shows the expanded $P_{(\gamma)}$ response.

(approx. 100mM buffer) in an isolated yield of 75% from ATP. The 5′-[^{16}O,^{17}O,^{18}O]AMP was re-chromatographed on the same system to remove residual traces of P_i.

Analysis

The chirality at the phosphorus atom of the 5′-[^{16}O,^{17}O,^{18}O]AMP derived from the polynucleotide kinase reaction was analysed by ^{31}P n.m.r. spectroscopy after cyclization and esterification as previously described (Jarvest *et al.*, 1980, 1981; Cullis *et al.*, 1981*a*).

^{31}P n.m.r. spectra were recorded on a Bruker WH300FT spectrometer with quadrature detection at 121.5MHz. The spectra are proton-noise-decoupled and the chemical shifts are measured with reference to external trimethyl phosphate; signals downfield from the reference are assigned positive chemical shifts.



Scheme 2. *Stereochemical course of [^{16}O,^{17}O,^{18}O]phosphoryl transfer catalysed by polynucleotide kinase (bacteriophage-T_4-infected E. coli B)*
◐ = ^{17}O; ● = ^{18}O.

Results and discussion

5′-[γ(*S*)-^{16}O,^{17}O,^{18}O]ATP (Lowe & Potter, 1981) was incubated with 3′-AMP and polynucleotide kinase (bacteriophage-T_4-infected *E. coli* B), and the

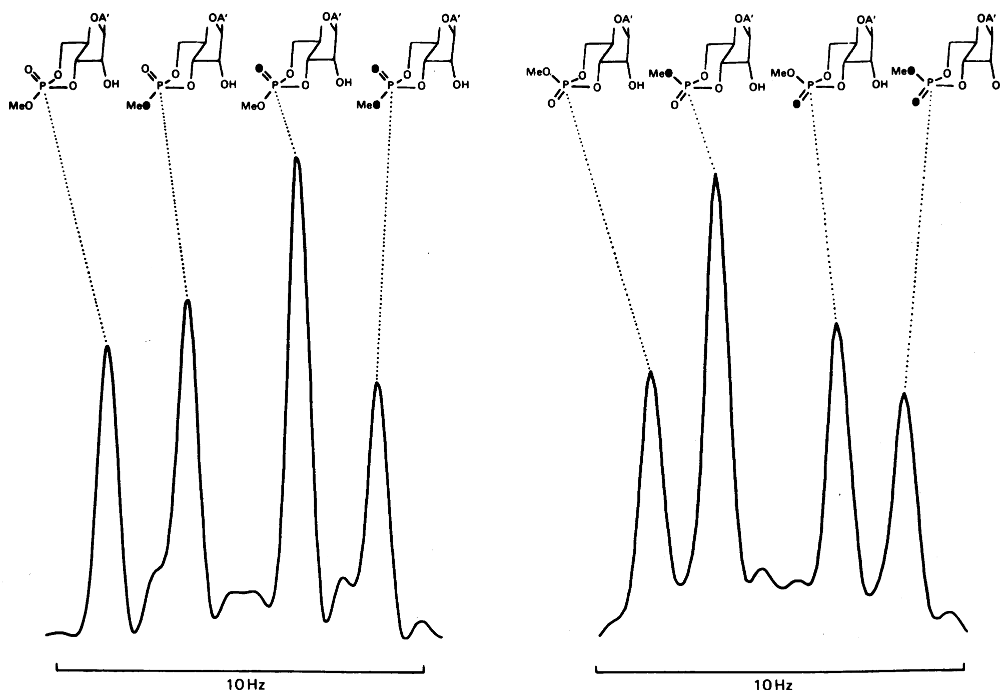


Fig. 2. *^{31}P n.m.r. spectrum (121.5MHz) in dimethyl sulphoxide (1.2ml) and [$^{3}H_3$]acetonitrile (1.0ml) of the equatorial and axial triesters derived by cyclization followed by methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP obtained by the polynucleotide kinase-catalysed transfer of the γ-phosphoryl group from 5′-[γ(S)-^{16}O,^{17}O,^{18}O]ATP*

The ^{31}P n.m.r. parameters are: offset 2200Hz, sweep width 2000Hz, acquisition time 2.05s, pulse width (angle), 15μs (75°), gaussian multiplication (line broadening −1.0Hz, gaussian broadening 0.3) in 8K and Fourier transform in 32K. A′ = *N*-1-methyladenine; ● = ^{18}O.

Table 1. *Observed relative peak intensities of the ^{31}P n.m.r. resonances (from Fig. 2) of the ^{18}O-labelled diastereoisomeric triesters derived by cyclization followed by methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP, and the calculated values expected from the known composition of the isotopically labelled ATP, for the polynucleotide kinase-catalysed phosphoryl transfer with retention and inversion of configuration*

● = ^{18}O.

	Equatorial triester			Axial triester		
		Calculated			Calculated	
	Observed	Retention	Inversion	Observed	Retention	Inversion
MeO–P=O	0.62	0.24	0.24	0.58	0.24	0.24
Me●–P=O	0.71	1.00	0.65	1.00	0.65	1.00
MeO–P=●	1.00	0.65	1.00	0.69	1.00	0.65
Me●–P=●	0.54	0.51	0.51	0.55	0.51	0.51

adenosine 3′,5′-[^{16}O,^{17}O,^{18}O]bisphosphate was selectively dephosphorylated to 5′-[^{16}O,^{17}O,^{18}O]AMP with nuclease P_1 (Scheme 2). In a preliminary experimental polynucleotide kinase was shown to have a half-life of about 29 h under the same reaction conditions. The 5′-[^{16}O,^{17}O,^{18}O]AMP was isolated by ion-exchange chromatography in 75% yield. The chirality at the phosphorus atom was analysed by ^{31}P n.m.r. spectroscopy after cyclization (which occurs with inversion of configuration) and esterification as described previously (Jarvest *et al.*, 1980, 1981; Cullis *et al.*, 1981*a*); the ^{31}P n.m.r. spectrum is shown in Fig. 2. The observed relative intensities of the isotopically labelled axial and equatorial triesters are compared in Table 1 with the expected ratios for phosphoryl transfer with retention and inversion of configuration, based on the known isotopic composition of the 5′-[$\gamma(S)$-^{16}O,^{17}O,^{18}O]-ATP (see the Materials and methods section). The unexpectedly large $^{16}O_2$–^{31}P n.m.r. signals presumably arise from some hydrolysis of ADP to AMP during the incubation period. However, this signal serves only as an internal reference, the relative intensity of the [$^{16}O_{eq.}$,$^{18}O_{ax.}$]- and [$^{18}O_{eq.}$,$^{16}O_{ax.}$]-triester signals providing the required stereochemical information. It is clear from these results that the phosphoryl transfer catalysed by polynucleotide kinase (bacteriophage-T_4-infected *E. coli* B) occurs with inversion of configuration at the phosphorus atom. The simplest interpretation of this result is that the phosphoryl group is transferred from ATP to the co-substrate by an ‘in-line’ mechanism. Thus polynucleotide kinase provides a further example of a phosphokinase that follows a sequential pathway and that catalyses phosphoryl transfer in the ternary complex with inversion of configuration at the phosphorus atom (Knowles, 1980; Cullis *et al.*, 1981*b*).

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