The stereochemical course of hydrolysis catalysed by snake venom 5'-nucleotide phosphodiesterase

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Adenosine 5'-(S)-[^{16}O , ^{17}O , ^{18}O]phosphate was pyrophosphorylated by the combined action of adenylate kinase and pyruvate kinase. The isotopomers of adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate were hydrolysed by venom 5'-nucleotide phosphodiesterase (*Crotalus adamanteus*) in H_2^{17}O . Analysis by ^{31}P nuclear magnetic resonance spectroscopy of the resulting adenosine 5'-[^{16}O , ^{17}O , ^{18}O]phosphate, after cyclization and esterification, showed that the hydrolysis occurs with retention of configuration at phosphorus. The most likely explanation of this observation is that the enzymic hydrolysis involves a double displacement at phosphorus with a covalent nucleotidyl–enzyme intermediate on the reaction pathway.

The stereochemical course of hydrolysis catalysed by venom 5'-nucleotide phosphodiesterase (EC 3.1.4.1) (*Crotalus adamanteus*) has been investigated recently using adenosine 5'-[1-thio]triphosphate (isomer B, R_p at P_α) (Bryant & Benkovic, 1979) and adenosine 5'-O-(R)-[^{18}O]phosphothioate-O-*p*-nitrophenyl ester (Burgers *et al.*, 1979a). No evidence had been reported previously for a nucleotidyl–enzyme intermediate with this enzyme, so that it was of interest that hydrolysis occurred with retention of configuration at phosphorus. This evidence does not rigorously prove the involvement of a nucleotidyl–enzyme intermediate, since adjacent attack by water followed by a pseudorotation would also give retention of configuration at phosphorus. However stereochemical studies with phosphokinases and phosphomutases (Knowles, 1980; Lowe *et al.*, 1981; Lowe & Potter, 1981a,b), suggest that enzymes catalyse individual phosphoryl transfer steps with inversion of configuration, making the double-displacement mechanism with a nucleotidyl–enzyme intermediate the most likely explanation.

Venom 5'-nucleotide phosphodiesterase is an enzyme we propose to use in connection with other stereochemical studies using substrates which are chiral at phosphorus due to isotopic substitution. It was essential therefore to determine the stereochemical course of hydrolysis by this enzyme with a chirally labelled phosphate diester.

Our approach was to pyrophosphorylate adenosine

5'-(S)-[^{16}O , ^{17}O , ^{18}O]phosphate by the combined action of adenylate kinase and pyruvate kinase. By using only a catalytic amount of ATP and an excess of phosphoenolpyruvate, the isotopomers of adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate should be formed with minimal isotopic dilution. The pyrophosphorylation will of course occur with equal probability at each of the three peripheral oxygens, the kinetic isotope effect being negligible (Scheme 1). Hydrolysis of the isotopomers by venom 5'-nucleotide phosphodiesterase should give isotopically labelled AMP which can be analysed for chirality at phosphorus by ^{31}P n.m.r. spectroscopy after cyclization and methylation (Jarvest *et al.*, 1981). By performing the hydrolysis in H_2^{17}O , and assuming for the moment complete isotopic enrichment, three isotopomers of AMP would be formed, only one of which is chiral (Scheme 1). The two achiral isotopomers however contain two ^{17}O atoms so that all the isotopomers derived from them by cyclization and methylation will contain at least one ^{17}O atom and their ^{31}P resonances will not be observed in the ^{31}P n.m.r. spectrum owing to the nuclear electric quadrupole moment of ^{17}O (Lowe *et al.*, 1979; Tsai, 1979; Tsai *et al.*, 1980). In practice of course the ^{17}O sites are not fully enriched, but since the isotopic composition will be known, the relative proportion of each isotopomer can be calculated for hydrolysis with retention and inversion of configuration, so that the stereochemical course of hydrolysis can be determined.

Scheme 1. Synthesis of the isotopomers of adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate and their hydrolysis by snake venom phosphodiesterase in H_2^{17}O with retention of configuration at phosphorus. O , ^{16}O ; $\oplus$, ^{17}O ; $\bullet$, ^{18}O .

Materials and methods

Adenosine 5'-(S)-[^{16}O , ^{17}O , ^{18}O]phosphate was prepared as described previously (Jarvest *et al.*, 1981). Bovine serum albumin, lactate dehydrogenase, adenylate kinase, pyruvate kinase and venom phosphodiesterase I (5'-exonuclease) from *Crotalus adamanteus* were obtained from Sigma; alcohol dehydrogenase was from Boehringer.

Venom phosphodiesterase I had an activity of 0.17 units/mg of protein with bis-(*p*-nitrophenyl)-phosphate as substrate.

Enzyme assays and u.v. measurements were performed on a Unicam SP 8-100 spectrophotometer. ^{31}P n.m.r. spectra were recorded on a Bruker WH 300 FT spectrometer with quadrature detection at 121.5 MHz; all spectra were proton noise decoupled.

Pyrophosphorylation of adenosine 5'-(S)-[^{16}O , ^{17}O , ^{18}O]phosphate

The reaction mixture consisted of Tris/HCl (42 mM, pH 8.0), KCl (60 mM), MgCl_2 (2.9 mM), adenosine 5'-(S)-[^{16}O , ^{17}O , ^{18}O]phosphate (1.4 mM, 600 μmol), ATP (0.008 mM, 3 μmol), phosphoenolpyruvate (3.6 mM), NADH (0.3 mM), bovine serum albumin (100 mg), yeast alcohol dehydrogenase (1200 units), lactate dehydrogenase (1000 units),

adenylate kinase (1500 units) and pyruvate kinase (1200 units) in water (415 ml) and ethanol (15 ml). The A_{260} of the solution fell sharply initially and eventually recovered to an equilibrium value at the termination of the reaction. EDTA (600 mg, 1.6 mmol) was then added and the solution was agitated with chloroform to denature the enzyme. The aqueous layer was applied to a column (40 ml) of DEAE-Sephadex A-25, and the column was eluted with a linear gradient of triethylammonium hydrogen carbonate (200–600 mM) over 24 h with a flow rate of 110 ml/h, collecting fractions over 12 min. The isotopomers of adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate were eluted in fractions 24–56 centred at 320 mM. The isolated yield was 0.53 mmol, 90%; the ^{31}P n.m.r. spectrum is shown in Fig. 1.

Hydrolysis of the isotopomers of adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate in H_2^{17}O by snake venom 5'-nucleotide phosphodiesterase

The isotopomers of adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate (100 μmol) were dissolved in H_2^{17}O (0.5 ml; isotopic composition 3.3 atom% ^{16}O , 43.5 atom% ^{17}O , 53.2 atom% ^{18}O). The solution was brought to pH 9.0 by addition of solid Tris. Snake venom 5'-nucleotide phosphodiesterase (0.4 units)

was added and the solution was incubated at 37°C. The pH value of the solution was maintained between 8.0 and 9.0 throughout the reaction by the addition of Tris. After 3 h the reaction was terminated by freezing and lyophilization to remove the $H_2^{17}O$. The residue was dissolved in water containing EDTA (1 mM) and the enzyme was denatured by vigorous agitation of the solution with chloroform. The aqueous layer was applied to a column (15 ml) of DEAE Sephadex A-25 and the column was eluted with a linear gradient of triethylammonium hydrogen carbonate (pH 8.0, 50–200 mM) over 24 h with a flow rate of 37 ml/h, collecting fractions over 20 min. The isotopomers of AMP were eluted in fractions 19–23, at about 85 mM buffer in a yield of 40 μ mol, 40%.

Analysis of the chirality of adenosine 5'-[^{16}O , ^{17}O , ^{18}O]phosphate

The isotopomers of the bis(triethylammonium) salt of AMP (40 μ mol) were converted to the monopyridinium salt by stirring with Dowex 50 (pyridinium form) in methanol/water (1:1, v/v) for 20 min. After filtration, the solvent was removed from the filtrate, and the residual glass was suspended in methanol to which was added trioctylamine (19 μ l, 40 μ mol) and the mixture was warmed until dissolution was complete. The solvent was removed and then dimethylformamide added and

evaporated thrice. The residue was then cyclized and methylated as previously described for adenosine 5'-(S)-[^{16}O , ^{17}O , ^{18}O]phosphate (Jarvest *et al.*, 1981).

Results and discussion

Adenosine 5'-(S)-[^{16}O , ^{17}O , ^{18}O]phosphate was synthesized by the general route for chiral [^{16}O , ^{17}O , ^{18}O]phosphate monoesters (Cullis & Lowe, 1981) as described previously (Jarvest *et al.*, 1981). The isotopic composition of the phosphorus [^{17}O]-oxychloride used in the synthesis was 3.3 atom% ^{16}O , 43.5 atom% ^{17}O , and 53.2 atom% ^{18}O (determined by mass spectrometry after conversion to trimethylphosphate), and the chiral purity of the (1*R*,2*S*)-1,2-[1- ^{18}O]dihydroxy-1,2-diphenylethane was also known (Lowe & Potter, 1981*a*). It was possible therefore to calculate the isotopomeric composition of the sample of adenosine 5'-(S)-[^{16}O , ^{17}O , ^{18}O]phosphate to be [$^{16}O_3$]AMP, 0.7%; [$^{16}O_2$, ^{18}O]AMP, 11.9%; (S)-[^{16}O , ^{17}O , ^{18}O]AMP, 31.7%; (R)-[^{16}O , ^{17}O , ^{18}O]AMP, 5.9%; [$^{16}O_2$, ^{17}O]AMP, 3.7%; [^{16}O , $^{18}O_2$]AMP, 46.1%. From these data the isotopomeric composition can be calculated for the adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate obtained by the random pyrophosphorylation of the peripheral oxygens by adenylate kinase and pyruvate kinase (Scheme 1). The ^{31}P n.m.r. spectrum (Fig. 1) of the isotopomers of adenosine

Fig. 1. ^{31}P n.m.r. spectrum (121.5 MHz) of the isotopomers of adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate in 2H_2O (50%) containing EDTA (1 mM) at pH 9.0

The expanded P_β resonances consist of two sets due to species with ^{16}O (low field) and ^{18}O (high field) in the P_α -O- P_β bridge. The expanded P_α resonances consist of five sets due to the following species (low to high field): [P_β -O- $P_\alpha O_2$]ATP, [P_β - $\bullet$ - $P_\alpha O_2$]ATP, [P_β -O- $P_\alpha \bullet O$]ATP, [P_β - $\bullet$ - $P_\alpha \bullet O$]ATP, and [P_β -O- $P_\alpha \bullet O_2$]ATP. $\circ$, ^{16}O ; $\bullet$, ^{18}O . The ^{31}P n.m.r. parameters are: offset 900 Hz, sweep width 3000 Hz, acquisition time 1.36 s, pulse width (angle) 15 μ s (75°), gaussian multiplication (line broadening -1.5 Hz, gaussian broadening 0.4) in 8 K, and Fourier transform in 32 K. The insets show the expanded P_α and P_β resonances.

5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate confirms the relative abundance of those isotopomers which do not contain ^{17}O .

Although it is possible to obtain the stereochemical information by hydrolysis of the isotopomers of adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate in H_2^{16}O , there is an advantage in using H_2^{17}O for this purpose, since this will give greater discrimination in the ^{31}P n.m.r. analysis between the alternative stereochemical courses of hydrolysis. For this reason the isotopomers of adenosine 5'-[α - ^{16}O , ^{17}O , ^{18}O]triphosphate were hydrolysed by venom 5'-nucleoside phosphodiesterase in H_2^{17}O (3.3 atom% ^{16}O , 43.5 atom% ^{17}O , 53.2 atom% ^{18}O), and the percentage abundance of the isotopomers of AMP was calculated for hydrolysis with retention and inversion of configuration at phosphorus. The isotopomers of AMP were cyclized and methylated as described previously (Jarvest *et al.*, 1981) and the ^{31}P n.m.r. spectrum is shown in Fig. 2. The cyclization occurs with inversion of configuration at phosphorus (Jarvest *et al.*, 1981) and since the [$^{16}\text{O}_{\text{ax.}}$, $^{18}\text{O}_{\text{eq.}}$]triesters predominate over the [$^{18}\text{C}_{\text{ax.}}$, $^{16}\text{O}_{\text{eq.}}$]triesters, [$^{16}\text{O}_{\text{ax.}}$, $^{18}\text{O}_{\text{eq.}}$]cyclic AMP must have predominated over [$^{18}\text{O}_{\text{ax.}}$, $^{16}\text{O}_{\text{eq.}}$]cyclic AMP after cyclization, hence adenosine 5'-(S)-[^{16}O , ^{17}O , ^{18}O]-

phosphate was formed in the snake venom phosphodiesterase-catalysed hydrolysis, showing that hydrolysis occurs with retention of configuration at phosphorus. The calculated isotopomeric composition of the AMP formed in the enzymic hydrolysis (with retention or inversion of configuration) allows the relative intensities of the triester resonances to be calculated and compared with the observed values (Table 1). The observed relative intensities show good agreement with the calculated values for hydrolysis with retention of configuration at phosphorus. Although this observation is compatible with an adjacent attack of the phosphate diester by water followed by a pseudorotation, it seems more probable, for the reasons already given, that a double displacement mechanism operates with a covalent nucleotidyl-enzyme intermediate on the reaction pathway.

Snake venom 5'-nucleotide phosphodiesterase is a further example of an enzyme which transforms a thiophosphate analogue by the same stereochemical course as the natural phosphate ester. There are two other examples at the phosphate diester level, namely cyclic AMP phosphodiesterase (Burgers *et al.*, 1979b; Jarvest & Lowe, 1980; Cullis *et al.*, 1981) and adenylate cyclase (Gerlt *et al.*, 1980;

Fig. 2. ^{31}P n.m.r. spectrum (121.5 MHz) of the equatorial and axial triesters derived by cyclization and methylation of the isotopomers of AMP obtained by snake venom 5'-nucleotide phosphodiesterase-catalysed hydrolysis in H_2^{17}O of the isotopomers of ATP

O, ^{16}O ; ●, ^{18}O ; A', N¹-methyladenine. The solvent is dimethylsulphoxide/ $[\text{d}_6]$ dimethyl sulphoxide (1:1, v/v). The ^{31}P n.m.r. parameters are: offset 2200 Hz, sweep width 2000 Hz, acquisition time 2.05 s, pulse width (angle) 15 μs (75°) gaussian multiplication (line broadening -1.0 Hz, gaussian broadening 0.35) in 8 K, and Fourier transform in 32 K.

Table 1. Comparison of the observed relative peak intensities of the ^{31}P resonances taken from Fig. 2 with the calculated values expected for hydrolysis by venom 5'-nucleotide phosphodiesterase with retention and inversion of configuration at phosphorus

The isotopically labelled diastereoisomeric triesters are derived by cyclization followed by methylation of the adenosine 5'-[^{16}O , ^{17}O , ^{18}O]phosphate and its isotopomers. O, ^{16}O ; ●, ^{18}O .

Species	Equatorial triester			Axial triester		
	Observed	Calculated for:		Observed	Calculated for:	
		Retention	Inversion		Retention	Inversion
MeO—P=O	0.47	0.43	0.43	0.48	0.43	0.43
Me●—P=O	1.00	1.00	0.87	0.91	0.87	1.00
MeO—P=●	0.87	0.87	1.00	1.00	1.00	0.87
Me●—P=●	0.97	0.98	0.98	1.02	0.98	0.98

Coderre & Gerlt, 1980). In addition there are now four phosphokinases which have been shown to catalyse both thiophosphoryl and phosphoryl transfer with inversion of configuration, namely, glycerol kinase (Orr *et al.*, 1978; Blättler & Knowles, 1979; Pliura *et al.*, 1980), hexokinase (Orr *et al.*, 1978; Lowe *et al.*, 1981; Lowe & Potter, 1981a), pyruvate kinase (Orr *et al.*, 1978; Lowe *et al.*, 1981) and polynucleotide kinase (Bryant *et al.*, 1981; Jarvest & Lowe, 1981). It seems likely therefore that thiophosphate analogues will in general follow the same stereochemical course as does the natural substrate. However, now that the synthesis of chiral [^{16}O , ^{17}O , ^{18}O]phosphate esters is established and analytical methods for determining their chirality have been developed, the much greater rate of transformation of the natural substrate will probably make the use of chiral phosphates advantageous, especially for enzymes which are difficult to obtain.

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