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A stereochemical investigation of the hydrolysis of cyclic AMP and the (S_P)- and (R_P)-diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate by bovine heart and baker's-yeast cyclic AMP phosphodiesterases

Richard L. JARVEST,* Gordon LOWE,* Janina BARANIAK† and Wojciech J. STEC†
**The Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K., and †Polish Academy of Sciences, Centre of Molecular and Macromolecular Studies, 90-362 Lodz, Boczna 5, Poland*

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Bovine heart cyclic AMP phosphodiesterase, which has a requirement for Mg^{2+}, hydrolyses cyclic AMP with inversion of configuration at the phosphorus atom, but only the (S_P)-diastereoisomer of adenosine cyclic 3′:5′-phosphorothioate is hydrolysed by this enzyme. By contrast, the low-affinity yeast cyclic AMP phosphodiesterase, which contains tightly bound Zn^{2+}, hydrolyses both the (S_P)- and the (R_P)-diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate, the (R_P)-diastereoisomer being the preferred substrate under $V_{max.}$ conditions. Both of the diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate, as well as cyclic AMP, are hydrolysed with inversion of configuration at the phosphorus atom by the yeast enzyme. It is proposed that, with both enzymes, the bivalent metal ion co-ordinates with the phosphate residue of the substrate, and that hydrolysis is catalysed by a direct 'in-line' mechanism.

Cyclic AMP phosphodiesterase plays a key role in the control of the intracellular concentration of the regulatory molecule cyclic AMP. This nucleotide, which is a mediator of hormone action and a modulator of enzymic activity, is synthesized from ATP by adenylate cyclase in the plasma membrane of a target cell when, for example, adrenaline is bound to its receptor protein at the cell surface (Sutherland, 1972). Cyclic AMP phosphodiesterase catalyses the hydrolysis of cyclic AMP to AMP, so that when the hormone is bound to its receptor a steady-state concentration of cyclic AMP is maintained. When the hormone receptor is not occupied the adenylate cyclase activity is 'switched off', and the intracellular concentration of cyclic AMP falls rapidly owing to hydrolysis by cyclic AMP phosphodiesterase (Appleman *et al.*, 1973; Wells & Hardman, 1977).

Cyclic AMP phosphodiesterases exist as both low-affinity (K_m approx. 100μM for cyclic AMP) and high-affinity (K_m approx. 1μM for cyclic AMP) forms. The high-K_m enzyme is soluble and will hydrolyse several 3′:5′-cyclic nucleotides, whereas the low K_m enzyme often occurs in particulate or membranous fractions and is specific for cyclic AMP. The phosphodiesterases from most sources have a pH optimum between 7.5 and 8.5 and require a bivalent metal ion for optimal activity. Mg^{2+} and Mn^{2+} are about equally effective, but Zn^{2+} is a powerful inhibitor (Drummond & Yamamoto, 1971). However, the high-K_m cyclic AMP phosphodiesterase from yeast has no requirement for a free bivalent metal ion but contains tightly bound Zn^{2+} (Londesborough, 1978).

Although the function of the bivalent metal ion has not been established in cyclic AMP phosphodiesterases, it seems likely that it is involved in binding the substrate. In order to provide some evidence to substantiate this view, a stereochemical investigation of the hydrolysis of the (S_P)- and (R_P)-diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate with both bovine heart (Mg^{2+}-dependent) and yeast (Zn^{2+}-containing) phosphodiesterases was undertaken, in addition to a study of the stereochemical course of hydrolysis of isotopically labelled cyclic AMP.

Materials and methods

Materials

Cyclic AMP phosphodiesterase from bovine heart (0.17 unit/mg) was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) and from baker's yeast (approx. 85 units/A_{280} unit) by the method of Londesborough (1978). Bovine serum albumin, pyruvate kinase, adenylate kinase, lactate dehydrogenase, phosphoenolpyruvate and NADH were obtained from Sigma Chemical Co. ^{18}O isotope shifts on ^{31}P n.m.r. spectra were measured on a Bruker WH 300 FT spectrometer with quadrature

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detection at 121.5 MHz; all spectra are broad-band-proton-noise-decoupled. Enzyme assays and u.v. measurements were performed on a Unicam SP.8-100 spectrophotometer. pH measurements were made on a Radiometer PHM84 pH-meter.

[(S)-^{16}O,^{17}O,^{18}O]AMP and its cyclization to the isotopomers of cyclic AMP

The synthesis of [(S)-^{16}O,^{17}O,^{18}O]AMP and its cyclization to the isotopomers of cyclic AMP were performed as described by Jarvest *et al.* (1981). The [(S)-^{16}O,^{17}O,^{18}O]AMP was shown by ^{31}P n.m.r. spectroscopy to contain (proportions in parentheses) [$^{16}O_3$]AMP (0.04), [$^{16}O_2$,^{18}O]AMP (0.28) and [^{16}O,$^{18}O_2$]AMP (1.00) (isotopomers containing ^{17}O are not observed), and the products of cyclization to be in the proportions cyclic [$^{16}O_{eq.}$,$^{16}O_{ax.}$]-AMP (0.35), cyclic [$^{16}O_{eq.}$,$^{18}O_{ax.}$]AMP (0.75), cyclic [$^{18}O_{eq.}$,$^{16}O_{ax.}$]AMP (1.00) and cyclic [$^{18}O_{eq.}$,$^{18}O_{ax.}$]-AMP (0.55); from the known isotopic composition of the $H_2{}^{17}O$ used in the synthesis it can be calculated that the ^{17}O species will be present in the following relative proportions: cyclic [$^{16}O_{eq.}$,$^{17}O_{ax.}$]-AMP (0.45), cyclic [$^{17}O_{eq.}$,$^{16}O_{ax.}$]AMP (0.20), cyclic [$^{17}O_{eq.}$,$^{18}O_{ax.}$]AMP (0.32) and cyclic [$^{18}O_{eq.}$,$^{17}O_{ax.}$]-AMP (0.07). The proportions of the isotopomers of the cyclic AMP used in the experiment with yeast cyclic AMP phosphodiesterase were similarly calculated.

Adenosine cyclic 3′:5′-(S_P)-phosphorothioate and adenosine cyclic 3′:5′-(S_P)-phosphorothioate

These compounds were synthesized as described by Baraniak *et al.* (1979).

Hydrolysis of the isotopomers of cyclic AMP by bovine heart cyclic AMP phosphodiesterase in $H_2{}^{17}O$

To a solution of glycylglycine (2.8 mg, 21.5 μmol), magnesium acetate tetrahydrate (1.0 mg, 4.7 μmol) and the isotopomers of cyclic AMP (triethylammonium salt) [100 μmol; derived by cyclization of [(S)-^{16}O,^{17}O,^{18}O]AMP with diphenyl phosphorochloridate followed by potassium t-butoxide (Jarvest *et al.*, 1981)] in $H_2{}^{17}O$ (0.5 ml; 6.2 atom% ^{16}O, 42.2 atom% ^{17}O, 51.6 atom% ^{18}O) was added bovine heart cyclic AMP phosphodiesterase (4 mg, 0.8 unit) at 4°C, and the solution was incubated at 37°C, the pH being maintained between 7 and 8 by additions of Tris (five 3 mg portions). After 3.5 h, the reaction was terminated by freezing, and the excess $H_2{}^{17}O$ was recovered by freeze-drying on a vacuum line. The residue was dissolved in water and vigorously agitated with chloroform and EDTA (8 mg). The aqueous layer was applied to a column (9 ml) of DEAE-Sephadex A-25 and developed with a gradient of triethylammonium bicarbonate buffer (50–200 mM) at 22 ml/h over 24 h with collection of 15 min fractions. The bistriethylammonium salt of the isotopically labelled AMP was eluted in fractions 19–35 (approx. 90 mM buffer) and was obtained after removal of the buffer as a white glassy solid (85 μmol).

Hydrolysis of adenosine cyclic 3′:5′-(R_P)-phosphorothioate by yeast cyclic AMP phosphodiesterase in $H_2{}^{18}O$

Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (10.7 mg, 45 μmol) and bovine serum albumin (1 mg) were dissolved in water (1 ml), the solution was adjusted to pH 8.2 by addition of aqueous KOH and the mixture was freeze-dried. The residue was dissolved in $H_2{}^{18}O$ (0.5 ml; 99 atom% ^{18}O), and adenosine cyclic 3′:5′-(R_P)-phosphorothioate (triethylammonium salt) (13.4 mg, 30 μmol) and yeast cyclic AMP phosphodiesterase (0.27 ml; 1.8 units) were added. The solution was incubated at 37°C for 20 h and the pH was maintained in the range 7.2–8.2 by addition of solid Tris. The solution was frozen, and the $H_2{}^{18}O$ was recovered by freeze-drying on a vacuum line. The residue was taken up in water, and the enzyme was denatured by vigorous agitation with chloroform, the chloroform being twice further extracted with water.

Hydrolysis of adenosine cyclic 3′:5′-(S_P)-phosphorothioate by yeast cyclic AMP phosphodiesterase in $H_2{}^{18}O$

The reaction was performed as described for the (R_P)-isomer but with adenosine cyclic 3′:5′-(S_P)-phosphorothioate (triethylammonium salt) (4.5 mg, 10 μmol) in $H_2{}^{18}O$ (0.5 ml; 80 atom% ^{18}O).

Pyrophosphorylation of the samples of adenosine 5′-[^{18}O]phosphorothioate

The pyrophosphorylation of the samples of adenosine 5′-[^{18}O]phosphorothioate to adenosine 5′-[α-^{18}O][α-thio]triphosphate by using adenylate kinase and pyruvate kinase was performed by the method of Sheu & Frey (1977).

Hydrolysis of the isotopomers of cyclic AMP by yeast cyclic AMP phosphodiesterase in $H_2{}^{17}O$

The reaction was performed as described for adenosine cyclic 3′:5′-(R_P)-phosphorothioate but with the isotopomers of cyclic AMP [80 μmol; derived from adenosine 5′-[(S)-^{16}O, ^{17}O, ^{18}O]phosphate, (Jarvest *et al.*, 1981)] and yeast phosphodiesterase (50 μl; 0.33 unit) in $H_2{}^{17}O$ (0.5 ml; 37.3 atom% ^{16}O, 28.1 atom% ^{17}O, 34.6 atom% ^{18}O) over a period of 4.5 h. The product was applied to a column (10 ml) of DEAE-Sephadex A-25 and eluted with a gradient of triethylammonium bicarbonate buffer (50–200 mM) run at 25 ml/h over 24 h with collection of 20 min fractions. The bistriethylammonium salt of the isotopomers of AMP was eluted in

fractions 30–38 (115 mM buffer) and was isolated after removal of buffer (32 μmol, 40%).

Kinetics of the hydrolysis of (S_P)- and (R_P)-diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate by yeast and bovine heart cyclic AMP phosphodiesterases

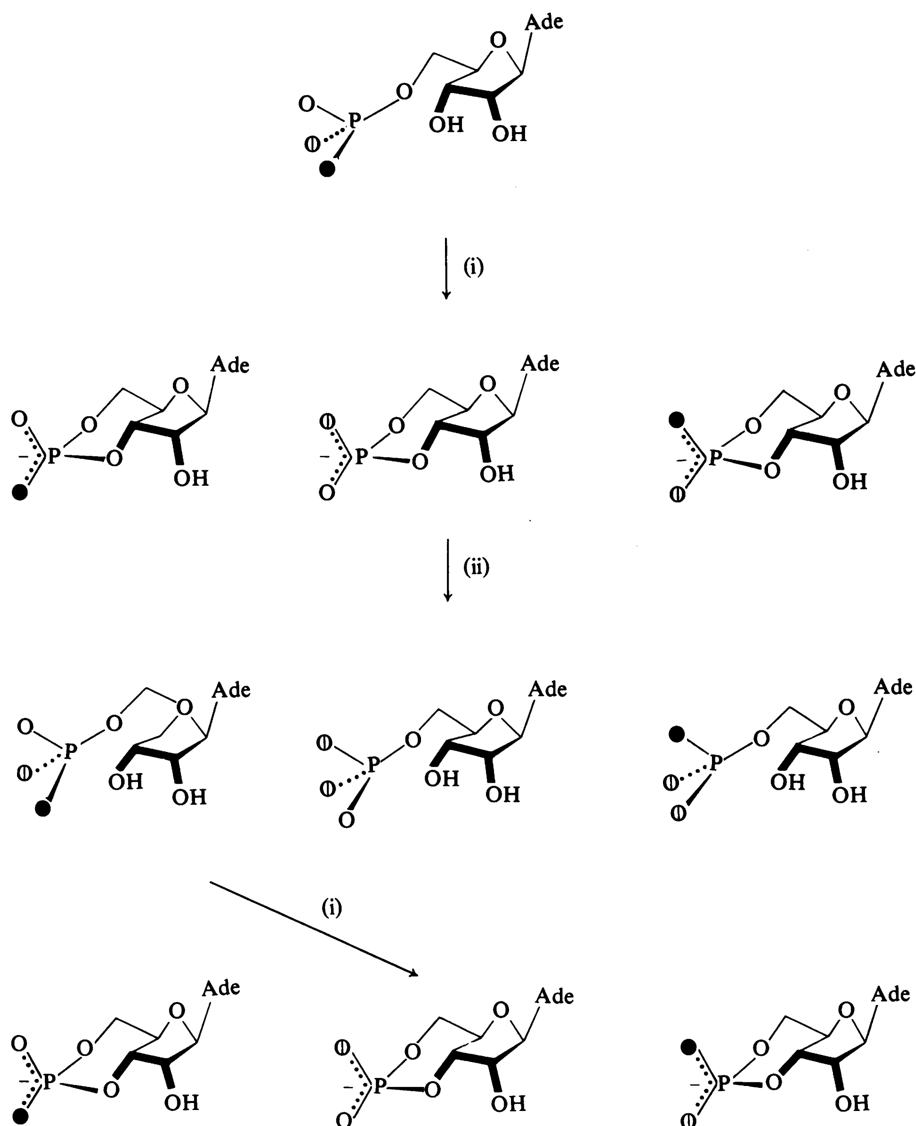
A 50 mM-Hepes/KOH solution, pH 7.5, containing bovine serum albumin (1 mg/ml) was employed as a buffer for the assays. Assays were performed at 37°C, and the hydrolysis was coupled to deamination by AMP deaminase, which was monitored at 265 nm in a 2 mm quartz cuvette [except that the most concentrated solution of the (*R*)-isomer was monitored in a 1 mm cuvette]. To adenosine cyclic 3′:5′-phosphorothioate isomers at various concentrations (0.02–0.82 mM) in buffer (1 ml) was added the deaminase (50 μl; 0.3 unit), followed by yeast phosphodiesterase (10 μl; 0.07 unit). To cyclic AMP at various concentrations (0.05–0.42 mM) in buffer (2 ml) was added yeast phosphodiesterase (10 μl; 0.07 unit), and to this solution (1 ml) was added the deaminase (50 μl; 0.3 unit).

To establish whether the diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate were substrates for the bovine heart enzyme, the (R_P)-diastereoisomer (0.7 mM) and the (S_P)-diastereoisomer (0.5 mM) were incubated with the bovine heart cyclic AMP phosphodiesterase (3 mg) and were assayed under the same conditions as described for the yeast enzyme.

Results and discussion

[(*S*)-^{16}O,^{17}O,^{18}O]AMP was synthesized by our general method of synthesis (Cullis & Lowe, 1978, 1981) and converted into the isotopomers of cyclic AMP by treatment with diphenyl phosphorochloridate followed by potassium t-butoxide, as shown in Scheme 1. The cyclization has been shown to proceed stereospecifically, with inversion of configuration at the phosphorus atom (Jarvest *et al.*, 1981). In a preliminary experiment cyclic AMP phosphodiesterase was shown to hydrolyse cyclic AMP to AMP in $H_2{}^{18}O$ with the incorporation of just 1 ^{18}O atom per molecule, i.e. the enzyme does not catalyse ^{18}O exchange. If the [(*S*)-^{16}O,^{17}O,^{18}O]-AMP were fully enriched at the isotopically labelled sites, then only the cyclic [^{16}O,^{18}O]AMP would give chiral [^{16}O,^{17}O,^{18}O]AMP on hydrolysis with cyclic AMP phosphodiesterase in fully enriched $H_2{}^{17}O$, as shown in Scheme 1. (N.B. The hydrolysis is shown as proceeding with inversion of configuration, as is subsequently proven: see below.) The achiral isotopomers of AMP would contain 2 ^{17}O atoms per molecule, and the products must retain at least 1 ^{17}O atom per molecule on recyclization (and methylation), and so would not appear in the ^{31}P n.m.r. spectrum (and are not shown in Scheme 1), owing to the nuclear electric quadrupolar relaxation caused by ^{17}O (Lowe *et al.*, 1979; Tsai, 1979; Tsai *et al.*, 1980). In practice, of course, the ^{17}O sites shown in Scheme 1 are not fully enriched, but, since the isotopic compositions of the phosphorus [^{17}O]-oxychloride (3.3 atom% ^{16}O, 43.5 atom% ^{17}O and 53.2 atom% ^{18}O) and (1*R*,2*S*)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane (94 atom% ^{18}O) used in the synthesis of [(*S*)-^{16}O,^{17}O,^{18}O]AMP are known, it is possible to calculate the relative proportion of the isotopomers of cyclic AMP, knowing that the cyclization proceeds with inversion of configuration (Jarvest *et al.*, 1981). The relative proportions of [$^{16}O_2$]-, [$^{16}O_{eq.}$,$^{18}O_{ax.}$]-, [$^{18}O_{eq.}$,$^{16}O_{ax.}$]- and [$^{18}O_2$]-isotopomers of cyclic AMP were confirmed by ^{31}P n.m.r. spectroscopy (see the Materials and methods section). Moreover, since the isotopic composition of the [^{17}O]water used for the hydrolysis of the isotopomers of cyclic AMP by cyclic AMP phosphodiesterase is known, the relative proportion of each isotopomer of AMP expected for hydrolysis with retention and inversion of configuration can be calculated. The isotopomers of AMP obtained by hydrolysis with cyclic AMP phosphodiesterase in [^{17}O]water were cyclized and methylated as described previously (Jarvest *et al.*, 1981); the ^{31}P n.m.r. spectrum is shown in Fig. 1. Because the ^{17}O sites are not fully enriched, four axial and four equatorial triester resonances are observed that are due to the [$^{16}O_2$]-, [$^{16}O_{ax.}$,$^{18}O_{eq.}$]-, [$^{18}O_{ax.}$,$^{16}O_{eq.}$]- and [$^{18}O_2$]-isotopomers. The stereochemical course of the hydrolysis is determined by the relative proportion of the cyclic [$^{16}O_{ax.}$,$^{18}O_{eq.}$]AMP to the cyclic [$^{18}O_{ax.}$,$^{16}O_{eq.}$]AMP from which the diastereoisomeric methyl esters are derived; if the former predominates the reaction must have occurred with inversion of configuration, whereas if the latter predominates the reaction must have occurred with retention of configuration. The ^{31}P n.m.r. spectrum (Fig. 1) shows that for both the axial and equatorial triesters the cyclic [$^{16}O_{ax.}$,$^{18}O_{eq.}$]AMP methyl ester predominates over the cyclic [$^{18}O_{ax.}$,$^{16}O_{eq.}$]AMP methyl ester, and so the hydrolysis has proceeded with inversion of configuration. The observed relative intensities from Fig. 1 are compared with the calculated values for retention and inversion of configuration in Table 1, providing a quantitative assessment of the stereospecificity of the hydrolysis. A preliminary communication of this work has been reported (Jarvest & Lowe, 1980; Cullis *et al.*, 1981).

Bovine heart cyclic AMP phosphodiesterase hydrolyses adenosine cyclic 3′:5′-(S_P)-phosphorothioate in $H_2{}^{18}O$ also with inversion of configuration at the phosphorus atom (Burgers *et al.*, 1979). We have found, however, that bovine heart cyclic AMP phosphodiesterase does not hydrolyse adenosine



Scheme 1. *Method adopted for determining the stereochemical course of cyclic AMP phosphodiesterase hydrolysis by using the isotopomers of cyclic AMP formed from* [(*S*)-[16]*O*,[17]*O*,[18]*O*]*AMP by cyclization with inversion of configuration at the phosphorus atom*

The hydrolysis in $H_2{}^{17}O$ is shown as occurring with inversion of configuration, which was proved by recyclization (with inversion of configuration) and analysis by ^{31}P n.m.r. spectroscopy after methylation. In order to simplify the presentation the ^{17}O and ^{18}O sites are shown fully enriched, although in practice this is not so (see the text). Reagents: (i) (*a*) $(PhO)_2POCl$, $Bu^n{}_3N$; (*b*) Bu^tOK in Me_2N–CHO; (ii) cyclic AMP phosphodiesterase in $H_2{}^{17}O$. ◐, ^{17}O; ●, ^{18}O.

cyclic 3′:5′-(R_P)-phosphorothioate (at a rate that can be measured), in contrast with a report that the (S_P)- and (R_P)-diastereoisomers are about equally good substrates (Eckstein *et al.*, 1974). [Professor Eckstein now considers that the adenosine cyclic 3′:5′-phosphorothioate used was predominantly the (S_P)-diastereoisomer and not a mixture of the (S_P)- and (R_P)-diastereoisomers as previously thought, and has confirmed our observation that only the (S_P)-diastereoisomer is a substrate (personal communication).]

Yeast cyclic AMP phosphodiesterase hydrolyses

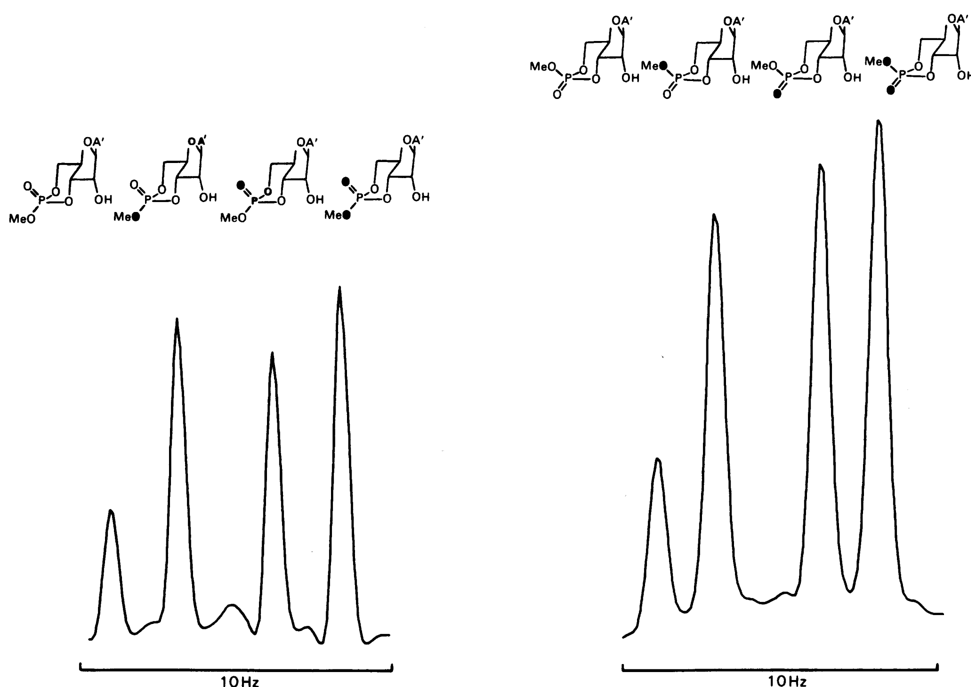


Fig. 1. *^{31}P n.m.r. spectrum (121.5 MHz) of the equatorial and axial triesters derived by cyclization and methylation of the isotopomers of AMP obtained by bovine heart cyclic AMP phosphodiesterase-catalysed hydrolysis in $H_2{}^{17}O$ of the isotopomers of cyclic AMP derived by stereospecific cyclization of [(S)-^{16}O,^{17}O,^{18}O]AMP*

O, ^{16}O; ●, ^{18}O; A′, N^1-methyladenine. The solvent is dimethyl sulphoxide/[$^2H_6$]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 (70°), broadband proton decoupling, gaussian multiplication (line broadening −0.9 Hz, gaussian broadening 0.4) in 8 K and Fourier transform in 32 K.

●

Table 1. *Comparison of the observed relative peak intensities of the ^{31}P nuclear magnetic resonances from Fig. 1 with the calculated values for hydrolysis of the isotopomers of cyclic AMP by bovine heart cyclic AMP phosphodiesterase in $H_2{}^{17}O$ with retention and inversion of configuration at the phosphorus atom*

●, ^{18}O.

	Equatorial triester			Axial triester		
	Observed	Calculated		Observed	Calculated	
		Retention	Inversion		Retention	Inversion
MeO–P=O	0.44	0.39	0.39	0.45	0.39	0.39
Me●–P=O	1.00	0.89	1.00	0.91	1.00	0.89
MeO–P=●	0.90	1.00	0.89	1.00	0.89	1.00
Me●–P=●	1.09	1.07	1.07	1.08	1.07	1.07

both the (S_P)- and the (R_P)-diastereoisomers of adenosine cyclic 3′ : 5′-phosphorothioate; the kinetic parameters are shown in Table 2, and are compared with those of cyclic AMP. $V_{max.}/K_m$ values for the diastereoisomers are very similar, and are about two orders of magnitude smaller than those for cyclic AMP, in accord with expectation, since thiophosphate esters are intrinsically less reactive than phosphate esters towards substitution at the phosphorus atom. Interestingly, the (R_P)-diastereoiso-

mer has a K_m comparable with that of cyclic AMP, whereas the (S_P)-diastereoisomer binds more tightly, and thus under conditions of substrate saturation ($V_{max.}$) the (R_P)-diastereoisomer is the preferred substrate.

The behaviour of the diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate is strikingly similar to the behaviour of the A- and B-diastereoisomers of adenosine [β-thio]triphosphate as a substrate for hexokinase in the presence of Mg^{2+} and Zn^{2+} (Jaffe & Cohn, 1979). In the presence of Mg^{2+} the B-diastereoisomer is overwhelmingly favoured ($V_{max.,B}/V_{max.,A} = 590$), whereas in the presence of Zn^{2+} the two diastereoisomers are about equally active [$V_{max.,B}/V_{max.,A} = 1.6$; $(V_{max.}/K_m)_B/(V_{max.}/K_m)_A = 0.8$]; with this enzyme the metal ion binds to the βγ-residues of adenosine [β-thio]triphosphate (Cornelius & Cleland, 1978). The specificity of the Mg^{2+}-dependent bovine heart cyclic AMP phosphodiesterase for adenosine cyclic 3′:5′-(S_P)-phosphorothioate and the similar kinetic parameters of the Zn^{2+}-dependent yeast cyclic AMP phosphodiesterase for the (S_P)- and (R_P)-diastereoisomers [$V_{max.(R_P)}/V_{max.(S_P)} = 3.6$; $(V_{max.}/K_m)_{(R_P)}/(V_{max.}/K_m)_{(S_P)} = 0.9$] is neatly accounted for if the metal ion is co-ordinated to the phosphate moiety of the substrate in both enzymes.

In spite of the extensive use of phosphorothioate esters as stereochemical probes, no opportunity has previously been taken to demonstrate that both diastereoisomers of a phosphorothioate diester are catalysed by a given enzyme with the same stereochemical consequences. Clearly, if they follow different stereochemical pathways, the use of phosphorothioates could give misleading stereochemical information. Thus the (R_P)- and the (S_P-diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate were hydrolysed by yeast cyclic AMP phosphodiesterase in $H_2{}^{18}O$ (Scheme 2). The adenosine 5′-[^{18}O]phosphorothioate obtained from each diastereoisomer was pyrophosphorylated by the combined action of adenylate kinase and pyruvate kinase (Sheu & Frey, 1977), and the position of the ^{18}O was determined by ^{31}P n.m.r. spectroscopy, in the way first used to determine the absolute configuration of the diastereoisomers of adenosine [α-thio]triphosphate (Jarvest & Lowe, 1979).

The ^{31}P n.m.r. spectrum of the product derived from the (R_P)-diastereoisomer of adenosine cyclic 3′:5′-phosphorothioate is shown in Fig. 2. The ratio of adenosine [(S_P)-α-thio]triphosphate to adenosine [^{18}O][(S_P)-α-thio]triphosphate is 0.66 and corresponds exactly to the composition of the labelled water used in the hydrolysis. An isotope shift is apparent on both the $P_{(\alpha)}$ and $P_{(\beta)}$ resonances, indicating that the ^{18}O atom is in the bridging position and hence that the enzyme-catalysed hydrolysis has occurred with inversion of configuration at the phosphorus atom. The ^{31}P n.m.r. spectrum of the product derived from the (S_P)-diastereoisomer of adenosine cyclic 3′:5′-phosphorothioate is shown in Fig. 3. Again the ratio of adenosine [(S_P)-α-thio]triphosphate to adenosine [^{18}O][(S_P)-α-thio]triphosphate (0.98) corresponds exactly to the isotopic composition of the water, but in this case an isotope shift only appears on the $P_{(\alpha)}$ resonance. The ^{18}O atom is thus in the non-bridging position on $P_{(\alpha)}$, indicating that hydrolysis has occurred with inversion of configuration at the phosphorus atom. It is gratifying to observe that both diastereoisomers follow the same stereochemical course.

To establish that the adenosine cyclic 3′:5′-phosphorothioate diastereoisomers follow the same stereochemical course as the natural substrate cyclic AMP with the yeast cyclic AMP phosphodiesterase, the isotopomers of cyclic AMP derived by stereospecific cyclization of [(S)-^{16}O,^{17}O,^{18}O]AMP (Jarvest *et al.*, 1981) were hydrolysed by the yeast enzyme in $H_2{}^{17}O$, and the product was isolated and analysed as described for the bovine heart enzyme (Scheme 1). The ^{31}P n.m.r. spectrum obtained is shown in Fig. 4. As with the bovine heart enzyme, the axial and equatorial triesters derived from cyclic [$^{16}O_{ax.}$,$^{18}O_{eq.}$]AMP predominate over those derived from cyclic [$^{18}O_{ax.}$,$^{16}O_{eq.}$]AMP, indicating that the hydrolysis has occurred with inversion of configuration at the phosphorus atom. The observed relative intensities from Fig. 4 are compared with the values calculated for retention and inversion of configuration (Table 3). The finding that the diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate and the isotopically labelled cyclic AMP are all hydrolysed with inversion of configuration at the phosphorus atom provides reassuring evidence that phosphorothioate analogues are not likely to give misleading evidence about the stereochemical course of enzyme-catalysed reactions. These observations nicely complement the finding that the adenylate cyclase-catalysed formation of cyclic AMP from ATP and adenosine cyclic 3′:5′-(R_P)-phosphorothioate from adenosine [(S_P)-α-thio]triphosphate also proceed with inversion of configuration (Coderre & Gerlt, 1980; Gerlt *et al.*, 1980; Eckstein *et al.*, 1981).

The simplest interpretation of the stereochemical observations with bovine heart and yeast cyclic AMP phosphodiesterases is that hydrolysis of cyclic AMP and the adenosine cyclic 3′:5′-phosphorothioate diastereoisomers is catalysed by a direct 'in-line' mechanism with the bivalent metal ion binding to at least one of the exocyclic phosphate oxygen atoms; the intervention of a nucleotidyl-enzyme intermediate is effectively excluded.

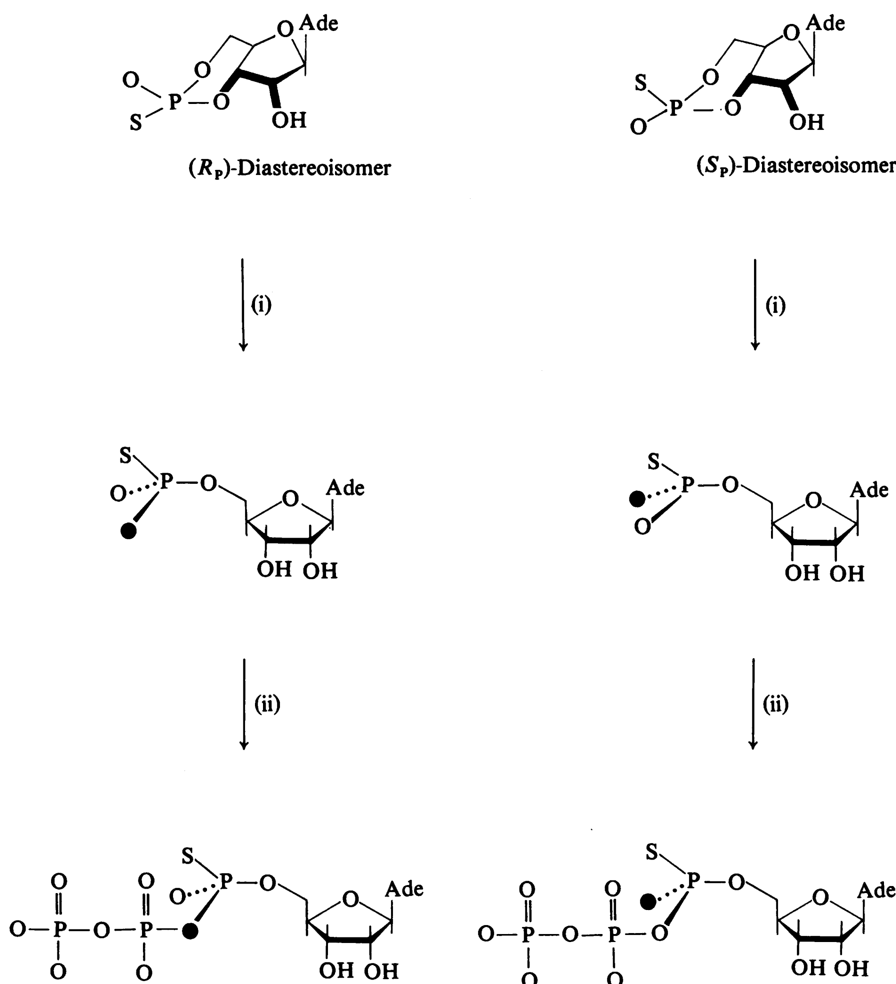
A mechanism that incorporates the stereochemical evidence, together with possible roles for the bivalent metal ion, is shown in Scheme 3. In

Table 2. *Kinetic parameters of yeast cyclic AMP phosphodiesterase-catalysed hydrolysis of cyclic AMP and the* (S_P)- *and* (R_P)-*diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate* (*cyclic AMPS*)

The kinetics were determined at 37°C in 50mM-Hepes/KOH buffer, pH 7.5, containing bovine serum albumin (1 mg/ml).

	Cyclic AMP*	Cyclic (R_P)-AMPS	Cyclic (S_P)-AMPS
$V_{max.}$ (μmol·min⁻¹·mg of protein⁻¹)	66 ± 11	0.68 ± 0.04	0.19 ± 0.01
K_m (μM)	130 ± 40	112 ± 12	28.4 ± 2.4
$V_{max.}/K_m$ (min⁻¹·mg of protein⁻¹)	0.54 ± 0.09	0.0061 ± 0.0003	0.0066 ± 0.0003

* At pH 7.7 and 30°C; Londesborough & Lukkari (1980) found $K_m = 122 \pm 6\,\mu M$.



Scheme 2. *Hydrolysis of the diastereoisomers of adenosine cyclic 3′:5′-phosphorothioate by yeast cyclic AMP phosphodiesterase in* $H_2{}^{18}O$

The hydrolysis is shown as occurring with inversion of configuration at the phosphorus atom, which was proved by converting each adenosine [^{18}O]phosphorothioate into adenosine [^{18}O][(S_P)-α-thio]triphosphate enzymically and determining by ^{31}P n.m.r. spectroscopy whether the ^{18}O was bonded to $P_{(\alpha)}$ alone or was in the $P_{(\alpha)}$–O–$P_{(\beta)}$ bridge. Reagents: (i) yeast cyclic AMP phosphodiesterase in $H_2{}^{18}O$; (ii) adenylate kinase, ATP, Mg^{2+}; pyruvate kinase, phosphoenolpyruvate, K^+, lactate dehydrogenase and NADH. ●, ^{18}O.

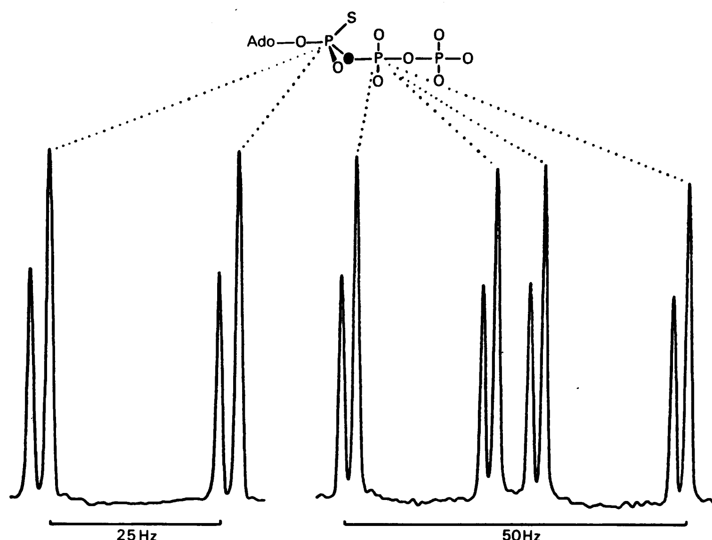


Fig. 2. *^{31}P n.m.r. spectrum (121.5 MHz) of $P_{(\alpha)}$ and of $P_{(\beta)}$ adenosine [(S_P)-α-thio]triphosphate obtained by enzymic pyrophosphorylation of the adenosine 5′-[^{18}O]phosphorothioate derived by yeast cyclic AMP phosphodiesterase-catalysed hydrolysis of adenosine cyclic 3′:5′-(R_P)-phosphorothioate in $H_2{}^{18}O$ (66 atom% ^{18}O)*

The spectrum was obtained in 50 atom% ^2H_2O, pH 9.0, containing EDTA (1 mM). The ^{31}P n.m.r. parameters are: offset −350 Hz (for $P_{(\beta)}$) or 7800 Hz (for $P_{(\alpha)}$), sweep width 400 Hz, acquisition time 1.28 s, pulse angle 70°, gaussian multiplication (line broadening −1.1 Hz, gaussian broadening 0.35) in 1 K and Fourier transform in 16 K.

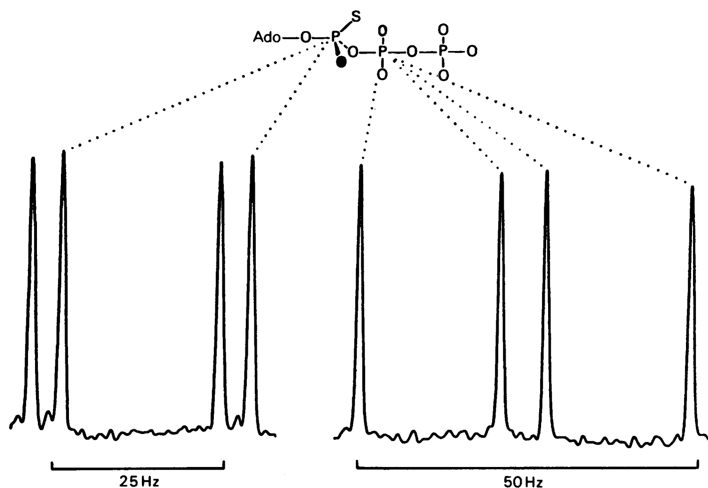


Fig. 3. *^{31}P n.m.r. spectrum (121.5 MHz) of $P_{(\alpha)}$ and $P_{(\beta)}$ of adenosine [(S_P)-α-thio]triphosphate obtained by enzymic pyrophosphorylation of the adenosine 5′-[^{18}O]phosphorothioate derived by yeast cyclic AMP phosphodiesterase-catalysed hydrolysis of adenosine cyclic 3′:5′-(S_P)-phosphorothioate in $H_2{}^{18}O$ (50 atom% ^{18}O)*

The solvent and ^{31}P n.m.r. parameters were the same as in Fig. 2.

addition to co-ordinating to the phosphate moiety of the substrate, the bivalent metal ion is almost certain to be co-ordinating a water molecule or hydroxide ion. Although a general base-catalysed hydrolytic mechanism has not been excluded, a metal-ion-co-ordinated hydroxide ion would be ideally situ-

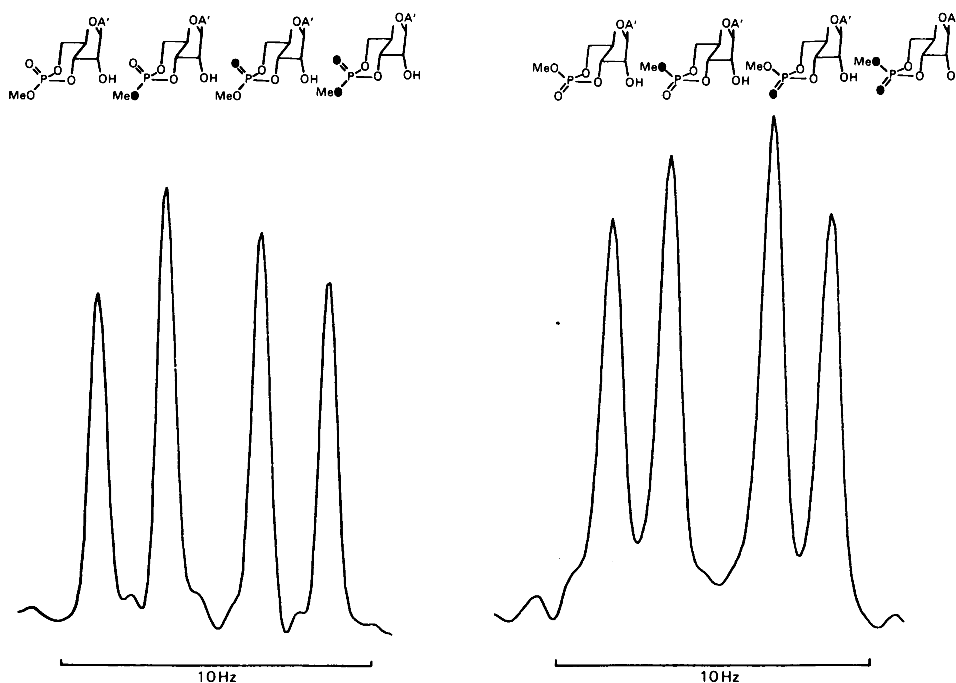


Fig. 4. *^{31}P n.m.r. spectrum (121.5 MHz) of the equatorial and axial triesters derived by cyclization and methylation of the isotopomers of AMP obtained by yeast cyclic AMP phosphodiesterase-catalysed hydrolysis in $H_2{}^{17}O$ of the isotopomers of cyclic AMP derived by stereospecific cyclization of* [(*S*)-^{16}O, ^{17}O, ^{18}O]*AMP*

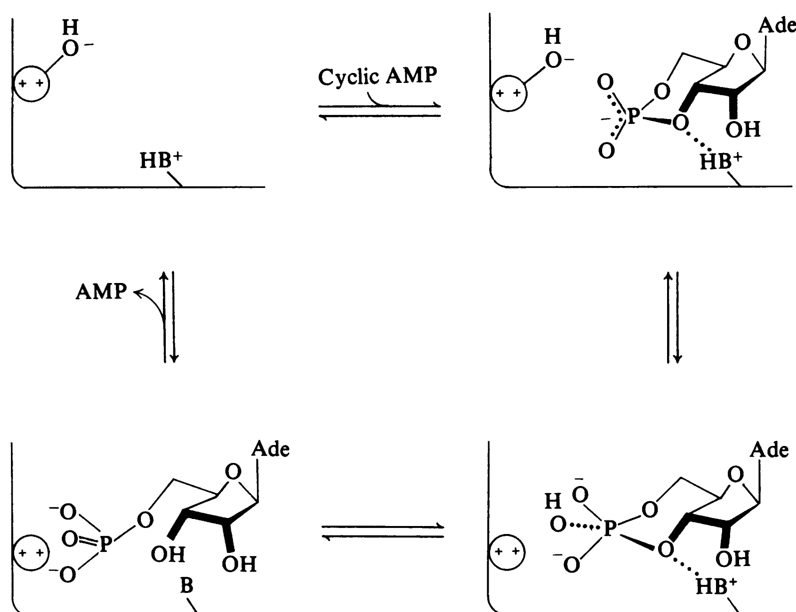
O, ^{16}O; ●, ^{18}O; A′, N^1-methyladenine. The solvent is dimethyl sulphoxide/[$^2H_6$]dimethyl sulphoxide (1 : 1, v/v). The ^{31}P n.m.r. parameters are: offset 2200 Hz, sweep width 2000 Hz, acquisition time 2.05 s, pulse width (angle) 15 μs (70°), broadband proton decoupling, gaussian multiplication (line broadening − 1.0 Hz, gaussian broadening 0.3) in 8 K and Fourier transform in 32 K.

Table 3. *Comparison of the observed relative peak intensities of the ^{31}P nuclear magnetic resonances from Fig. 4 with the calculated values for hydrolysis of the isotopomers of cyclic AMP by yeast cyclic AMP phosphodiesterase in $H_2{}^{17}O$ with retention and inversion of configuration at the phosphorus atom*

●, ^{18}O.

	Equatorial triester			Axial triester		
	Observed	Calculated		Observed	Calculated	
		Retention	Inversion		Retention	Inversion
MeO–P=O	0.75	0.72	0.72	0.79	0.72	0.72
Me●–P=O	1.00	0.87	1.00	0.92	1.00	0.87
MeO–P=●	0.90	1.00	0.87	1.00	0.87	1.00
Me●–P=●	0.79	0.72	0.72	0.80	0.72	0.72

ated to be an effective hydrolytic agent for a displacement reaction at the phosphorus atom. The transient trigonal bipyramidal intermediate (or transition state) with two negatively charged equatorial oxygens would be stabilized by the bivalent metal ion and would be expected to collapse to AMP by general acid catalysis. Although some features of this proposal are without direct experimental sup-



Scheme 3. *Possible mechanism for the hydrolysis of cyclic AMP by bovine heart and baker's-yeast cyclic AMP phosphodiesterases, illustrating the electrostatic interaction between the bivalent metal ion ((++), Mg^{2+} and Zn^{2+} respectively) with the substrate and the 'in-line' displacement at the phosphorus atom*

The involvement of a metal ion co-ordinated hydroxyl ion and a general acid catalyst seem likely but are not proven.

port, the geometic constraints provided by an 'in-line' displacement, the rigid structure of the substrate and the direct involvement of the bivalent metal ion severely limit the range of mechanistic possibilities.

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