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Mechanisms of the ATPase-like Activity of the Macrocyclic Polyamine Receptor Molecule $[24]N_6O_2$

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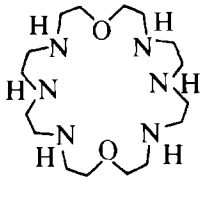
The macrocyclic polyamine 1,4,7,13,16,19-hexaaza-10,22-dioxatetraeicosan ($[24]N_6O_2$) which catalyzes the hydrolysis of ATP to ADP and P_i, at least in part via the phosphorylated macrocycle, with a rate enhancement of about 100, has been shown to catalyze positional isotope exchange in $[\beta\text{-}^{18}O_2]$ATP in the presence of Ca^{2+} although not in its absence. It also catalyzes the hydrolysis of ATPγS, but only with a rate enhancement of 3. No evidence could be found for thiophosphorylation of the macrocycle and the hydrolysis of adenosine 5′[γ(*R*)-^{17}O,^{18}O,thio]triphosphate was shown to proceed with inversion of configuration at phosphorus. It appears that the macrocycle is capable of catalyzing at least two hydrolytic pathways, ATPγS following a direct "in line" displacement mechanism with water.

INTRODUCTION

Macrocyclic polyamines form a group of synthetic receptors which, when protonated, bind a number of inorganic and organic ions, including phosphates and nucleotides (*1–3*). An exciting development in the chemistry of macrocyclic polyammonium ions was the discovery that 1,4,7,13,16,19-hexaaza-10,22-dioxatetraeicosan ($[24]N_6O_2$), **1**, in addition to forming tight complexes with nucleotides, catalyzes the hydrolysis of ATP (*4, 5*). The rate of hydrolysis is accelerated by a factor of about 100, with smaller enhancements in the rate of ADP and pyrophosphate hydrolysis. **1** was also found to catalyze the hydrolysis of acetyl phosphate, but in contrast to the hydrolysis of nucleotides, a substantial product (ca. 30%) of



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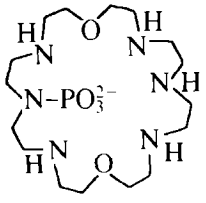
this reaction was pyrophosphate, demonstrating that the macrocycle could catalyze synthetic as well as hydrolytic reactions (*6*). This synthetic ability was extended when it was shown that Ca^{2+}, in addition to enhancing the catalysis of ATP hydrolysis by **1**, also induces the synthesis of pyrophosphate during this reaction (*7*).

The precise mode of binding of nucleotides to **1** is at present unknown, but for the sake of illustration the bound complex has been represented as showing binding to P_α and P_γ of ATP. From the results of pH-metric titrations, "stability constants" for **1** · ATP complexes in different states of protonation were calculated (*3, 4*). However, it must be recognized that each of these "complexes" is a mixture of complexes differing in its specific interactions between the nucleotide and the macrocycle.

A number of factors are believed to contribute to the catalytic properties of **1.** In the hydrolysis of ATP and acetyl phosphate between pH 7 and 8.5 a resonance at δ_P 10 in the NMR spectra of reaction mixtures has been assigned to the phosphorylated macrocycle **2,** and this species has been shown to be kinetically com-



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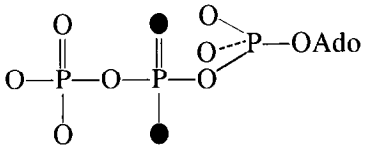
petent in acidic, as well as neutral solution (*4, 5*). This suggests that **1** can act as a nucleophilic catalyst. A value for the entropy of activation for the macrocycle-catalyzed ATP hydrolysis of -10 ± 1 EU has been taken to indicate an associative mechanism. However, Blackburn *et al.*, on the basis of studies on the macrocycle-catalyzed hydrolysis of a number of methylene bisphosphonate analogs of nucleotides, concluded that the reaction to form **2** is dissociative in character (*8*). It is likely that **1** may also serve as a general acid catalyst, enhancing the leaving-group capacity of ADP by the transfer of a proton from one of the ammonium ions. Unprotonated amino groups, present in all macrocycle · ATP complexes at neutral pH, may also act as general base catalysts for incoming nucleophiles. Electrostatic catalysis, by neutralizing negative charge on the polyphosphate chain and so allowing the approach of negatively charged electron-rich nucleophiles, is also expected to be an important contributor to the overall catalysis (*4, 5*).

RESULTS AND DISCUSSION

Positional isotope exchange studies. In order to study whether intermediate **2** is able to rephosphorylate ADP, and to further investigate the effects of Ca^{2+} on the

1-catalyzed hydrolysis of ATP, a positional isotope exchange study was undertaken. The macrocycle was incubated, with and without an equivalent of Ca^{2+}, with one equivalent of [β-$^{18}O_2$]ATP **3** (● = ^{18}O) (*9*). Both reactions were allowed



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to proceed at pH 7.6 and 70°C to the same point (approximately 60% hydrolysis) before the reaction was quenched and the ATP isolated and analyzed by ^{31}P NMR spectroscopy. The reaction mixture with Ca^{2+} present was divided into two aliquots after termination of the reaction. The first portion was purified immediately while the second was acidified to pH 4.5 and allowed to stand at room temperature for 24 h, as described by Yohannes *et al.* (*7*). The percentage positional isotope exchange in the ATP recovered from each reaction is shown in Table 1.

From the results shown in Table 1 it is clear that, in the absence of Ca^{2+}, ADP does not attack **2,** unless this reverse reaction is rapid with respect to rotation about the $O_{\alpha\beta}$–P_β bond, which seems unlikely. The observation supports a mechanism in which only water attacks **2** to regenerate the free macrocycle in the absence of Ca^{2+}.

In contrast, when Ca^{2+} is present, phosphates as well as water can be phosphorylated by **2.** Yohannes *et al.* (*7*) noted that the presence of Ca^{2+} increased the rate of disappearance of ATP but decreased the rate of hydrolysis of **2,** such that after 80% of the initial ATP had reacted 50% of the phosphate derived from the breakdown of ATP was present as the phosphorylated intermediate. Although no pyrophosphate was formed at pH 7.6, even after 80% of the ATP had reacted, when the reaction mixture was adjusted to pH 4.5 and left to stand overnight pyrophosphate formation was observed. The results of the positional isotope

TABLE 1

The Percentage Positional Isotope Exchange (PIX) Observed after 60% Hydrolysis of [β-$^{18}O_2$]ATP Catalyzed by [24]N_6O_2

[[24]N_6O_2]/mM	[β-$^{18}O_2$ATP]/mM	[$CaBr_2$]/mM	PIX(%)
10	10	0	0
10	10	10	6[a]
10	10	10	10[b]

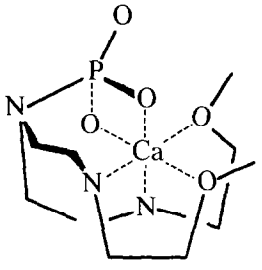
[a] ATP isolated immediately after reaction at 70°C.

[b] Reaction mixture adjusted to pH 4.5 and allowed to stand at room temperature for 24 h before isolation of the ATP.

exchange experiments show that at pH 7.6 substantial reaction of ADP with **2** can occur. The effect of the pH adjustment in the experiment described by Yohannes *et al.* (*7*) is to dissolve precipitated calcium phosphate which builds up during reaction at pH 7.6. The additional amount of positional isotope exchange observed after the adjustment of the reaction mixture to pH 4.5 is due to further reaction of ADP with the large amount of **2** which accumulates during reaction at neutral pH. This has all reacted at pH 4.5 within 24 h at room temperature because of the enhanced reactivity of phosphoroamidates in acidic media (*5, 10*). It should be noted that although the reaction has been allowed to proceed, albeit at a very much lower temperature and at a different pH, for a further 24 h during this experiment, the hydrolysis of ATP has proceeded by only an additional 5%. Unless there has been a very significant change in the reaction mechanism on the reduction in the temperature and the pH, this difference in the endpoint of the reaction cannot explain the large difference in the degree of positional isotope exchange which is observed. Rather, this must be due in large part to the attack of ADP on **2,** the synthesis of ATP partially balancing the hydrolysis of ATP which will continue at a reduced rate at 20°C. It has been found recently that ATP is formed by reaction of ADP with **2** in dimethyl sulfoxide/water solution (*11*).

In attempting to rationalize the influence of Ca^{2+} on the reactions of **2,** the following data concerning the reactions catalyzed by [24]N_6O_2 must be accounted for. No pyrophosphate formation and no positional isotope exchange are observed in the reaction with ATP alone, while pyrophosphate formation is observed in the reactions with acetyl phosphate and Ca · ATP, together with positional isotope exchange in the latter case. Yohannes *et al.* (*7*) suggested that the effects of Ca^{2+} on the **1**-catalyzed hydrolysis of ATP are, in addition to accelerating the rate of ATP hydrolysis, to reduce the rate of hydrolysis of **2** by stabilization of the P–N bond and to disrupt the complexation of nucleotides with the macrocycle. In support of this ^{13}C NMR data were cited in which the shifts of the macrocycle and the macrocycle in the presence of Ca · ATP were nearly identical, while those of **1** in the presence of ATP differ significantly. This is in contrast to the work of Kimura *et al.*, who show that the presence of Ca^{2+} does not affect the amount of nucleotide available for complexation and that ternary macrocycle–nucleotide–metal ion complexes are the norm (*2*). An alternative explanation is that Ca^{2+}, which is known to be able to bind to polyamine macrocycles alone (*12*) exerts its effect solely by binding to **2.** Ca^{2+} is known to have a predilection for oxygen ligands relative to nitrogen ligands and it may be that the presence of the two ether linkages within the macrocycle enhance this binding. A possible structure for the Ca · **2** complex is shown in **4.** The effect of this complexation would be to increase electrostatic catalysis and lower the barrier to attack by ADP on **2.**

On the basis of this proposal, and from the knowledge that the addition of ions such as ADP or oxalate to the reaction mixture is known to inhibit pyrophosphate formation during the hydrolysis of acetyl phosphate, the reactions catalyzed by **1** may be explained as follows. In the reaction with acetyl phosphate, binding of phosphate to **2** is far stronger than binding of acetate, with attack by phosphate to give pyrophosphate proceeding at a rate comparable to that of the hydrolysis of **2.** In the reaction of **1** with ATP, the ADP binds better to **2** than does phosphate, so



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preventing formation of pyrophosphate, but electrostatic repulsion prevents attack by ADP on **2.** In the presence of Ca^{2+} the electrostatic barrier is reduced, allowing positional isotope exchange to occur, but the same effect allows the rate of phosphate attack on **2** to increase markedly such that the formation of pyrophosphate may now be observed, in spite of the low concentration of the $P_i \cdot$ **2** complex. The enhanced binding of ions to **2** in the presence of Ca^{2+} will sterically hinder attack by water so that **2** accumulates.

The [24]N_6O_2-catalyzed hydrolysis of ATPγS. A stereochemical investigation of the reaction catalyzed by **1** was of considerable interest. In order to obtain information concerning the stereochemical course of reactions in which orthophosphate is produced from ATP it is necessary to use as a substrate ATPγS made chiral at P_γ by isotopic substitution.

A kinetic study was undertaken first in order to examine the effect of [24]N_6O_2 on the rate of hydrolysis of ATPγS. Since inorganic thiophosphate hydrolyzes rapidly in solutions below pH 7 all reactions were carried out at pH 8. While kinetic studies were usually conducted by Hosseini *et al.* (*4, 5*) without buffering and Blackburn *et al.* (*8*) report that in their unbuffered experiments at pH 6.7 the pH never varied by more than 3%, in a trial experiment in which **1** and ATPγS were allowed to react at pH 8.0 without buffering, the pH had changed by more than 1 pH unit by the end of the reaction. All subsequent reactions were buffered in 100 mM Tris · HCl solutions at pH 8.0. Plots of $-\ln([ATP\gamma S]_t/[ATP\gamma S]_o)$ against time are shown in Fig. 1 and values for the first-order rate constants are shown in Table 2.

TABLE 2

Rate Constants for the Hydrolysis of ATPγS at pH 8.0 and 70°C

[ATPγS]/mM	[[24]N_6O_2]mM	($k \times 10^3$)/min
10	0	14 ± 1
10	10	38 ± 1

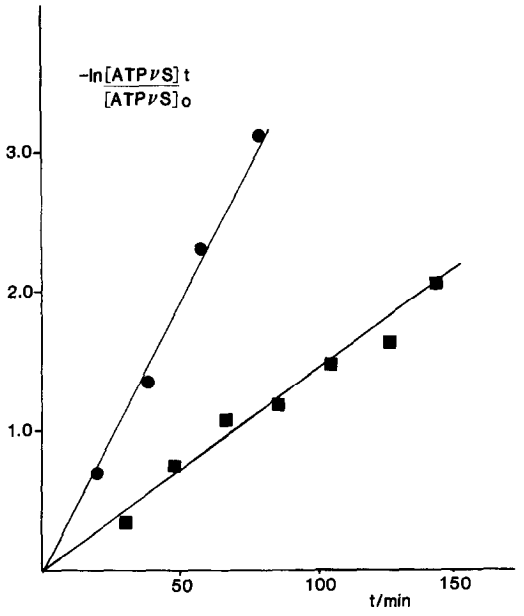

FIG. 1. Plots of $-\ln([ATP\gamma S]_t/[ATP\gamma S]_0)$ against time for the hydrolysis of 1 mM ATPγS at pH 8.0 and 70°C; ● with 1 mM [24]N_6O_2 and ■ with no addition.

The results show a small but significant enhancement of the rate of ATPγS hydrolysis by the macrocycle. A number of factors may be responsible for a lowering of the catalytic activity of **1** with ATPγS relative to that shown with ATP:

(i) The **1**-catalyzed hydrolysis of ATP at neutral pH is retarded in solutions of high ionic strength and specifically by chloride ions (*5*). The presence of 100 mM Tris · HCl buffer may be a cause of the diminution of catalysis.

(ii) The uncatalyzed hydrolysis of ATPγS is an order of magnitude faster than the uncatalyzed hydrolysis of ATP (*13*). It is likely that the macrocycle is less able to catalyze such a fast reaction. (The rate of hydrolysis of acetyl phosphate catalyzed by **1** at 40°C is 0.035 min^{-1}, only a fivefold rate enhancement over the uncatalyzed reaction (*6*) although this may also be due to poorer binding of acetyl phosphate to **1**.)

(iii) The chemical differences between ATP and ATPγS may result in different modes of binding of the two nucleotides. In particular the differences in charge distribution at P_γ may disturb the binding of ATPγS to the macrocycle. The sulfur in ATPγS carries one negative charge, while the charge density on the terminal oxygen atoms is consequently reduced (*14*). Assuming that the P–S and P–O bond orders are not altered by interaction with **1**, in complexes in which the P–S bond pointed away from the macrocycle, the electrostatic binding energy would be reduced relative to the binding of ATP. On the other hand in complexes in which the sulfur points toward the macrocycle, its increased size and its inability to form strong hydrogen bonds, particularly important at neutral pH where fewer of the

amino groups are protonated, may adversely affect the binding or alter the position of P_γ with respect to **1.**

(iv) The reduced rates of enzyme-catalyzed reactions of phosphorothioate monoesters relative to the rates of the natural phosphate monester substrates, inverting the order of rates observed for uncatalyzed hydrolysis, have been taken to indicate that enzymes do not catalyze the reactions via a free-energy pathway which favors mechanisms with a strong dissociative character by which the uncatalyzed reactions proceed (*15, 16*). If the macrocycle favors associative rather than dissociative mechanisms, then a similar effect may be operating.

^{31}P NMR experiments were performed in an attempt to observe the thiophosphate analog of **2.** Reactions containing 20 mM **1** and ATPγS in an NMR tube were followed by ^{31}P NMR spectroscopy at 202 MHz. In four experiments, all at pH 8, no signal for a transiently formed phosphorothioamidate was observed. Either no such intermediate exists or it is hydrolyzed at a rate faster than that at which it is formed.

The stereochemical experiment was then performed. Adenosine 5′-[γ(*R*)-^{17}O,^{18}O,thio]triphosphate (25 μmol) (*17*) was heated at 70°C with one equivalent of **1** and the reaction followed by HPLC.

When the reaction was complete, the [^{16}O,^{17}O,^{18}O]thiophosphate was isolated and treated with (*S*)-2-iodo-1-phenylethanol. The (*S*)-1-phenylethanol-2*S*-[^{16}O,^{17}O,^{18}O]thiophosphate was cyclized with diphenyl phosphorochloridate which proceeds with inversion of the configuration at phosphorus (*18*). The isotopomers of the cyclic thiophosphate were methylated with diazomethane to give a mixture of the *trans*- and *cis*-methyl esters. The ^{31}P NMR spectrum of the isotopomeric mixture of *trans*- and *cis*-methyl esters reveals only those isotopomers containing ^{16}O and ^{18}O, since ^{17}O directly bonded to phosphorus causes broadening of the ^{31}P resonance by scalar relaxation (*19, 20*).

When ^{18}O is directly bonded to phosphorus an isotope shift to higher field is observed (*21*) the magnitude of which is related to the bond order. When ^{18}O is in a phosphorus oxygen double bond the isotope shift is approximately twice that in a phosphorus oxygen single bond (*19*). The three lowest intensity resonances in each quartet of isotopomers arise because the ^{17}O site is not fully enriched. Since the predominant isotopomer has ^{18}O in the P=O bond for the *trans*-isomer and in the P–OMe bond for the *cis*-isomer, the inorganic [^{16}O,^{17}O,^{18}O]thiophosphate has the (*S*)-configuration and hence the reaction has proceeded with inversion of configuration at phosphorus. The comparison of the observed relative peak intensities with those calculated (Table 3) clearly shows that the reaction proceeds with inversion of configuration (within experimental limits). Although this could imply adjacent attack on the hypothetical intermediate (the thiophosphorylated **1**) there is no precedent for such a mechanism with phosphate monoesters, or amides and the simplest interpretation, consistent with the absence of any phosphorothioamidate in the ^{31}P NMR spectrum of the reaction mixture, is that the thiophosphoryl group is transferred directly to water.

This result could be explained by proposing that **1** can catalyze the hydrolysis of nucleoside triphosphates by two mechanisms, the first involving nucleophilic catalysis via the intermediate **2,** the second in which direct attack by water occurs by

TABLE 3

The Observed Intensities of the 4-(*S*)-Phenyl-2-methoxy-2-oxo-1,3,2-thiaoxaphosphalanes Derived from the [^{16}O,^{17}O,^{18}O]-Thiophosphate Obtained from the [24]N_6O_2-Catalyzed Hydrolysis of [γ-(*R*)-^{17}O,^{18}O]ATPγS

	trans-ester			*cis*-ester		
		Calculated			Calculated	
	Observed	Inversion	Retention	Observed	Inversion	Retention
MeO—P=O	0.36	0.37	0.37	0.36	0.37	0.37
Me●—P=O	0.22	0.23	0.34	0.33	0.34	0.23
MeO—P=●	0.34	0.34	0.23	0.24	0.23	0.34
Me●—P=●	0.08	0.06	0.06	0.07	0.06	0.06

a mechanism which would almost certainly be preassociative. In both mechanisms electrostatic catalysis and general acid catalysis by **1** could contribute to the rate enhancement. While the evidence to support the intermediacy of **2** in the hydrolysis of ATP is incontrovertible, this is probably not the sole pathway (*4, 5*). Indeed one explanation for the anomalous value for the entropy of activation obtained for the **1**-catalyzed hydrolysis of ATP at pH 7.6 might be that two different mechanisms for hydrolysis are occurring simultaneously.

While it is possible that a contribution to the reaction product could have arisen by reaction of free adenosine 5′-[γ(*R*)-^{17}O,^{18}O,thio]triphosphate with water, which at pH 8.0 would be expected to proceed with inversion of configuration (*22*), a kinetic experiment in which two equivalents of ATPγS were combined with one equivalent of macrocycle yielded a value for the first order rate constant of 0.039 $\pm$ 0.001 min^{-1} (cf. data in Table 2) suggesting that ATPγS is bound tightly to the macrocycle before reaction and that after the reaction ADP is rapidly replaced by ATPγS. This would be consistent with the data on the stability constants for **1** · nucleotide complexes which are several orders of magnitude higher for the binding of triphosphates than for the binding of diphosphates. The explanation of why ATP and ATPγS behave so differently on the macrocycle must lie in their different chemical properties. Thiophosphoryl groups react more readily than phosphoryl groups by dissociative mechanisms (*16*) but less readily by associative mechanisms (*23*). Were the reaction involving nucleophilic catalysis to be associative in character, then the reaction would be slowed on going from phosphoryl to thiophosphoryl group transfer, possibly by as much as an order of magnitude. This would allow the competing mechanism of direct thiophosphoryl transfer to water, a reaction which proceeds via a dissociative-like mechanism, to become the dominant route for hydrolysis of ATPγS.

Since the conformations of **1** · nucleotide complexes have not been studied it is impossible to be certain whether or not ATPγS binds differently from ATP and if so whether such different conformations would be sufficient to alter the mechanism of catalysis. At neutral pH, where the macrocycle is less protonated, and

therefore where hydrogen bonding will be relatively more important, the binding of the oxygens of P_γ of ATP to the macrocycle will probably be very important. Subject therefore to this assumption, it is concluded, on the basis of this work, that the switch in what appears to be the dominant (but not necessarily exclusive) mechanism, observed between ATP and ATPγS, is probably due to the greater associative character of the reaction of **1** with P_γ of the nucleotides compared to the macrocycle-catalyzed direct reaction of water at P_γ.

EXPERIMENTAL

Chemicals. 1,4,7,13,16,19-Hexaaza-10,22-dioxocyclotetraeicosan hexahydrochloride (**1**·6 HCl) was prepared by the published method (*24*).

Instrumentation and methods. The pH of aqueous solutions was measured with a Radiometer PHM84 pH meter with a combination electrode.

Optical densities were obtained using a Pye-Unicam SP8-100 spectrophotometer.

^1H NMR spectra were recorded on a Bruker AM500 spectrometer at 500.13 MHz. ^{31}P NMR spectra were recorded on a Bruker AM500 spectrometer at 202.46 MHz using a phosphorus-dedicated probe. All chemical shifts are quoted as positive for resonances downfield from the appropriate reference. For ^{31}P spectra the reference was external trimethylphosphate in D_2O. Unless otherwise specified, high resolution ^{31}P NMR spectra were recorded in degassed $H_2O:D_2O$ (1:1) containing 2 mM EDTA and 20 mM Tris, pH 9.0.

Evaporation of solvent refers to evaporation at reduced pressure using a rotary evaporator.

All anion-exchange chromatography was carried out at +4°C. Phosphorothioate containing fractions were detected by spotting onto a silica TLC plate and spraying with palladium dichloride (1% in 1 M HCl). Phosphorothioates appear yellow or brown. Residual buffer salts were removed from compounds purified in this way by the addition and evaporation of aliquots of methanol (20 ml).

HPLC analysis of nucleotides was performed using an analytical Mono-Q anion exchange column. The sample loading was typically 60 nmol of nucleotide, and the column was eluted with a linear gradient of 10–1000 mM Tris · HCl (pH 8.0) at 20°C.

Positional isotope exchange of adenosine 5'-[β-$^{18}O_2$]triphosphate catalyzed by 1,4,7,13,16,19-hexaaza-10,22-dioxocyclotetraeicosan ***1.*** 1,4,7,13,16,19-Hexaaza-10,22-dioxocyclotetraeicosan hexahydrochloride (5.6 mg, 10 μmol) and adenosine 5'-[β-$^{18}O_2$]triphosphate were dissolved in water (1 ml). The solution was adjusted to pH 7.6 using 1 M potassium hydroxide solution. The solution was incubated at 70°C for 100 min and then cooled rapidly to 0°C. Fast protein liquid chromatography analysis showed that 60% of the [β-$^{18}O_2$]ATP had been hydrolyzed. The mixture was diluted and purified by ion exchange chromatography using a column of DEAE-Sephadex A-25 (20 ml) and a linear gradient of triethylammonium bicarbonate (100–600 mM, pH 8.0, 750 ml). The recovered adenosine 5'-[$^{18}O_2$]triphos-

phate (3.5 μmol) when analyzed by high resolution ^{31}P NMR spectroscopy showed no positional isotope exchange.

Positional isotope exchange of adenosine 5'-[β-$^{18}O_2$]triphosphate catalyzed by 1,4,7,13,16,19-hexaaza-10,22-dioxocyclotetraeicosan ***1*** *in the presence of calcium ions.* 1,4,7,13,16,19-Hexaaza-10,22-dioxocyclotetraeicosan hexahydrochloride (4.8 mg, 8.7 μmol), adenosine 5'-[β-$^{18}O_2$]triphosphate (5.8 mg, 8.7 μmol), and calcium dibromide (2.0 mg, 8.7 μmol) were dissolved in water (0.9 ml). The solution was adjusted to pH 7.6 with 1 M potassium hydroxide solution. The mixture was incubated at 70°C for 28 min and then cooled rapidly to 0°C. FPLC analysis showed that 56% of the ATP had been hydrolyzed. The cloudy solution was divided into two equal portions.

The first portion was diluted, excess EDTA (0.1 M, pH 8.2) was added, and the ATP was purified by ion-exchange chromatography using a column of DEAE-Sephadex A-25 (15 ml) and eluting with a linear gradient of triethylammonium bicarbonate (100–600 mM, pH 8.0, 750 ml). The isolated [$^{18}O_2$]ATP (1.7 μmmol) was analyzed by high resolution ^{31}P NMR spectroscopy.

The second portion was adjusted to pH 4.5 using 1 M hydrochloric acid, whereupon the precipitated calcium salts dissolved. The solution was kept at room temperature for 24 h. It was then diluted, excess EDTA (0.1 M, pH 8.2) was added, and the ATP was purified by ion-exchange chromatography, using a column of DEAE-Sephadex A-25 (15 ml), eluting with a linear gradient of triethylammonium bicarbonate (100–600 mM, pH 8.0, 75 ml). The recovered [$^{18}O_2$]ATP (1.5 μmol) was analyzed by high resolution ^{31}P NMR spectroscopy.

Hydrolysis of adenosine 5'-[γ(R)-^{17}O,^{18}O,thio]triphosphate catalyzed by 1,4,7,13,16,19-hexaaza-10,22-dioxocyclotetraeicosan ***1.*** Tris (0.303 g, 2.5 mmol) and 1,4,7,13,16,19-hexaaza-10,22-dioxocyclotetraeicosan hexahydrochloride (14 mg, 25 μmol) were dissolved in water (25 ml), and the solution was adjusted to pH 8.0 with concentrated hydrochloric acid. Adenosine 5'-[γ(*R*)-^{17}O,^{18}O,thio]triphosphate (25 μmol) was added and the resultant solution incubated at 70°C for 70 min. The solution was rapidly cooled to 5°C, diluted, and applied to a column of DEAE-Sephadex A-25 (15 ml). The column was eluted with a linear gradient of triethylammonium bicarbonate buffer (30–300 mM, pH 8.1, 750 ml). Fractions which reacted positively with the palladium chloride spray were combined and evaporated to give [^{16}O,^{17}O,^{18}O]thiophosphate bis(triethylammonium) salt (11.5 μmol, 46%). The chirality was determined by the method of Arnold *et al.* (*18*).

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