Polyammonium Macrocycles as Catalysts for Phosphoryl Transfer: The Evolution of an Enzyme Mimic

MATHIAS P. MERTES and KRISTIN BOWMAN MERTES*

Departments of Chemistry and Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045

Received May 7, 1990 (Revised Manuscript Received August 10, 1990)

Molecular recognition features the design of synthetic receptor molecules that have high affinity and specificity for substrate molecules. Work pioneered over the last 30 years by researchers in macrocyclic chemistry has illustrated the potential of such molecules in this area. The era began in the early 1960s, when Busch published the first paper describing the rational design of synthetic macrocycles with an eye to mimicking biological macrocycles such as the hemes.[1] Less than 10 years later the scope of the field widened beyond just mimicking nature's macrocycles, especially with the contributions of Cram with his cavitands,[2] Lehn with cryptands,[3] and Pederson with the crown ethers.[4] The naturally occurring cyclodextrins have also added scope to this area, although they are not truly "synthetic" macrocycles.[5] Hence, by the early 1970s, concepts such as anion recognition and ion transport became realities with the aid of macrocyclic molecules.

The initial goal in designing a molecular receptor is to achieve specificity for a given substrate, resulting in the formation of a high-affinity complex. Once that is in hand, a next step may be to promote chemical transformations within the complex such that a situation analogous to that found in the enzymes ensues. Hence, it is desirable to apply lessons learned from nature's catalysts, the enzymes, in the design and synthesis of artificial receptors that go beyond simple substrate recognition to the catalysis of chemical transformations.

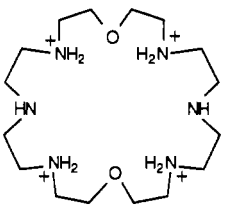
Enzymes use a number of ploys to enhance their efficiency. For example, both entropic and binding energy advantages of the enzyme–substrate complex serve as driving forces for the reactions. Furthermore, enzymes may utilize acid–base effects in general acid/general base catalysis. Electrostatic and covalent or nucleophilic catalysis may also serve to facilitate the process. Finally, the concept that an enzyme has the greatest affinity for the transition state of the reaction, nudging it toward completion, is evident in observations that strain and distortion also contribute to catalysis.

Table I
Properties of ATPases

$$ATP + H_2O \xrightarrow{ATPase/Mg^{2+}} ADP + P_i$$

process	transported	covalent intermediate
photophosphorylation	electrons	no
oxidative phosphorylation	protons	no
stomach gradient	K^+ out/H^+ in	yes
nerve gradient	Na^+ out/K^+ in	yes
intracellular storage	Ca^{2+} in	yes

The advantages of polyammonium macrocycles such as [24]N_6O_2 (1) as enzyme mimics are numerous. In the polyprotonated form they are soluble in water. The high positive charge density and potential hydrogen-bonding sites promote complex formation with biologically relevant anionic substrates. Furthermore, a common and versatile synthetic methodology is available for their preparation. This means that ring size can be readily varied in the synthetic scheme. Additionally both alkyl and aromatic groups can be incorporated in the ring, which allows for the construction of macrocycles with varying charge density (i.e., redox potential, hydrophobicity) at defined positions in the ring and for potential metal-binding sites. Pendant functionality can also be placed at virtually any position on the ring, or, as an extension of this aspect, polymacrocyclic rings can be constructed. By virtue of extensive design possibilities, limited only by the imagination, ditopic and higher order receptors designed to bind two or more different substrates are feasible. With these properties in mind, the historical account of how the polyammonium macrocycle, [24]N_6O_2 (1), was found to embody a number of the attributes of phosphoryl-transfer enzymes will serve to illustrate the broad range



1

Mathias P. Mertes was born in Chicago, IL, on April 22, 1932. He received his B.S. in pharmacy from the University of Illinois in 1954, his M.S. in medicinal chemistry from the University of Texas with C. O. Wilson in 1956, and his Ph.D. in medicinal chemistry from the University of Minnesota in 1960 with Ole Gisvold. From 1960 to 1989 he was on the medicinal chemistry faculty at the University of Kansas. His research activities included nucleic acid chemistry, inhibition of thymidylate synthetase, design and synthesis of selective glutamate receptor probes, and more recently, biomimetic macrocyclic chemistry.

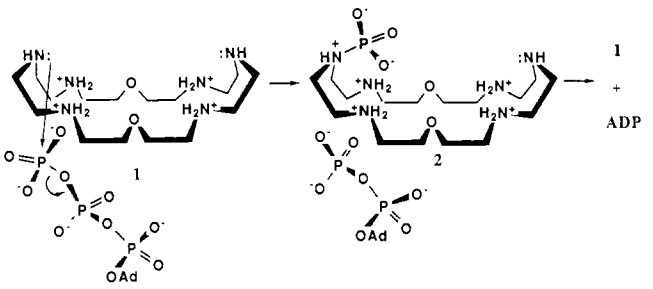
Matt's death in April 1989 meant the loss of an outstanding scientist, teacher, and human being. Matt was full of boundless energy, enthusiasm, and optimism for all aspects of life, which he readily transmitted to those around him. He was always ready to lend a sympathetic ear and was noted for his unselfish kindness and generosity. Matt was a scholar, colleague, and friend, who will always be remembered and is sorely missed by all who knew him.

Kristin Bowman Mertes was born in Philadelphia, PA, on June 4, 1946. She received her B.S. from Temple University in 1968 and her Ph.D. with Zvi Dori from Temple University in 1974. Following a postdoctoral appointment at The Ohio State University with Daryle Busch, she joined the faculty at the University of Kansas, where she is now Professor of Chemistry. Her research is in synthetic macrocycles as related to naturally occurring macrocycles such as the porphyrins, and as mimics for nonmacrocyclic enzymes.

(1) Thompson, M. C.; Busch, D. H. *Chem. Eng. News* **1962**, 57.
(2) Almy, J.; Garwood, D. C.; Cram, D. J. *J. Am. Chem. Soc.* **1973**, *95*, 2961–2962.
(3) (a) Dietrich, B.; Lehn, J.-M.; Sauvage, J.-P. *Tetrahedron Lett.* **1969**, 2885; (b) *Ibid.* **1969**, 2889.
(4) (a) Pedersen, C. J. *J. Am. Chem. Soc.* **1967**, *89*, 2459; (b) *Ibid.* **1967**, *89*, 7017.
(5) Breslow, R.; Overman, L. E. *J. Am. Chem. Soc.* **1970**, *92*, 1075.

0001-4842/90/0123-0413$02.50/0

Scheme I



of applicability of these molecules as enzyme mimics.

ATPase Mimicry

The affinity of polyammonium macrocycles for biologically relevant molecules such as adenosine triphosphate, ATP, was observed by Lehn in 1981.[6] Stability constants as high as 10^9 are observed for **1** and ATP, when the former is hexaprotonated. A logical extension of such findings was obviously toward mimicking the critical biological transformations in which ATP is involved.

ATP is ubiquitous in biological systems and is known as the "currency of energy", wherein the energy released by the hydrolytic cleavage of the terminal phosphate is coupled with numerous metabolically unfavorable reactions.[7] The molecule is relatively stable at pH 7.0, due to its high negative charge, but in the presence of the appropriate enzymes, the rate of hydrolysis increases by a factor of 10^{10}. Some of the characteristics of the ATPases, which constitute the class of enzymes known to hydrolyze ATP, are shown in Table I. Note that two important aspects of these reactions are the requirement for magnesium ion in all cases and the formation of a covalent enzyme–phosphoryl complex in several of the pathways. It is with respect to these characteristics, namely, metal ion effects and covalent catalysis, that the low molecular weight, polyammonium macrocycles can effectively (but not nearly as efficiently) mimic the naturally occurring ATPases.

Compared to a number of different polyammonium macrocycles, with varying degrees of affinity for ATP, the hexaazadioxa macrocycle $[24]N_6O_2$ (**1**) has been found to be one of the most efficient to date at catalyzing the dephosphorylation of ATP.[8] The first-order reaction rate for the dephosphorylation of ATP in the presence of **1** is readily monitored by ^{31}P NMR at pH 7, where the macrocycle is a mixture of primarily tetra- and pentaprotonated forms. The pK_{a3} for $[\mathbf{1}\cdot H_4]^{4+}$ ranges from 7.6 to 8.1, depending on the identity of the salt used to adjust ionic strength.[9,10] In addition to the anticipated products of adenosine diphosphate, ADP, and inorganic phosphate, P_i, a small amount of an intermediate phosphoramidate species, **2**, is observed, indicated by a fleeting resonance signal at 10 ppm in the ^{31}P NMR spectrum (Scheme I, Figure 1). Such a species results from covalent or nucleophilic catalysis: namely, by attack of the lone pair of the neutral central amine of **1** on the terminal γ-phosphate of ATP. The phosphoramidate **2** readily hydrolyzes to give inorganic phosphate and free macrocycle. As noted above, several ATPases function via a covalent enzyme–phosphoryl intermediate (Table I). Hence, nucleophilic catalysis, an enzyme ploy to gain efficiency, is also utilized by the mimic.

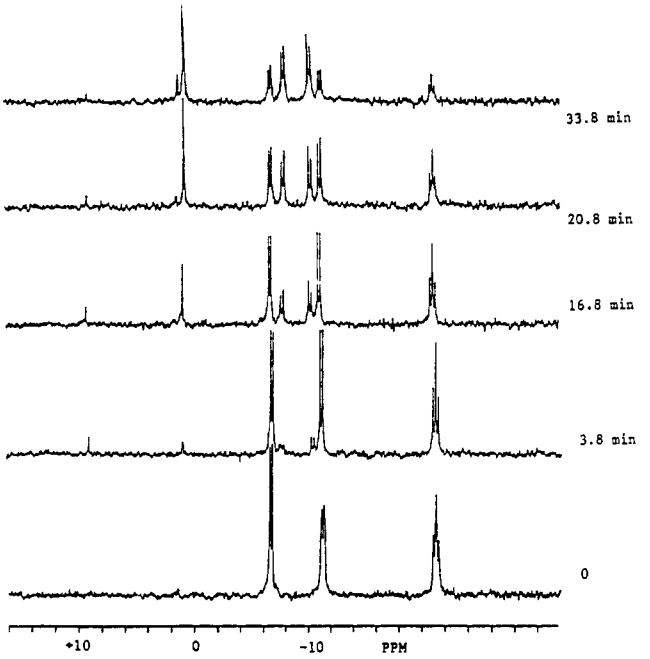


Figure 1. ^{31}P NMR spectra of ATP and $[24]N_6O_2$ (**1**) (0.03 M in each) in 10% D_2O/H_2O at pH 7.0 and 70 °C. The chemical shift assignments: ATP, $\alpha = -10.6$, $\beta = -20.6$, $\gamma = -5.7$; ADP, $\alpha = -9.2$, $\beta = -6.0$; inorganic phosphate (P_i), +2.5; AMP, +3.3; phosphoramidate, +10.3 ppm. The chemical shifts are relative to 0 ppm for 85% H_3PO_4 as an external standard (+, downfield).

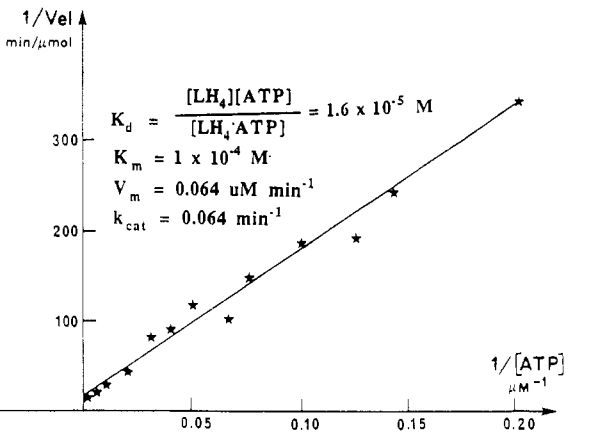


Figure 2. Double-reciprocal plot of velocity (Vel) versus 1/[ATP] in the reaction of excess ATP with 1×10^{-6} M $[24]N_6O_2$ (**1**) at pH 7 and 70 °C. Velocities are corrected for water hydrolysis of ATP.

Since this system shows reversible binding prior to the catalytic step, the Michaelis–Menten mechanism can be applied (eqs 1–3, Figure 2).[11] Lineweaver–Burk analysis of kinetic results using a 1 μM concentration of **1** gives $K_m = 1 \times 10^{-4}$ M, $V_m = 0.064$ μmol/min, and

(6) Dietrich, B.; Hosseini, M. W.; Lehn, J.-M.; Sessions, R. B. *J. Am. Chem. Soc.* **1981**, *103*, 1282.
(7) Walsh, C. F. *Enzymatic Reaction Mechanism*; W. H. Freeman: San Francisco, CA, 1979.
(8) (a) Hosseini, M. W.; Lehn, J.-M.; Mertes, M. P. *Helv. Chim. Acta* **1983**, *66*, 2454–2466. (b) Hosseini, M. W.; Lehn, J.-M.; Maggiora, L.; Mertes, K. B.; Mertes, M. P. *J. Am. Chem. Soc.* **1987**, *109*, 537–544.
(9) Hosseini, M. W. Ph.D. Dissertation, Université Louis Pasteur, Strasbourg, France.
(10) Motekaitis, R. J.; Martell, A. E.; Lecomte, J.-P.; Lehn, J.-M. *Inorg. Chem.* **1983**, *22*, 609–614.
(11) Hosseini, M. W.; Lehn, J.-M.; Jones, K. C.; Plute, K. E.; Mertes, K. B.; Mertes, M. P. *J. Am. Chem. Soc.* **1989**, *111*, 6330–6335.

Table II
Comparison of ATPase- and Macrocycle-Catalyzed Dephosphorylation of ATP Using Lineweaver-Burk Kinetics

catalyst	K_m, M	V_m, μM/min	k_{cat}, min^{-1}	k_{cat}/K_m
none			2.5×10^{-4}	
ATPase	1×10^{-4}		3.2×10^{4}	3.2×10^{8}
1	1×10^{-4}	0.064	6.4×10^{-2}	6.4×10^{2}

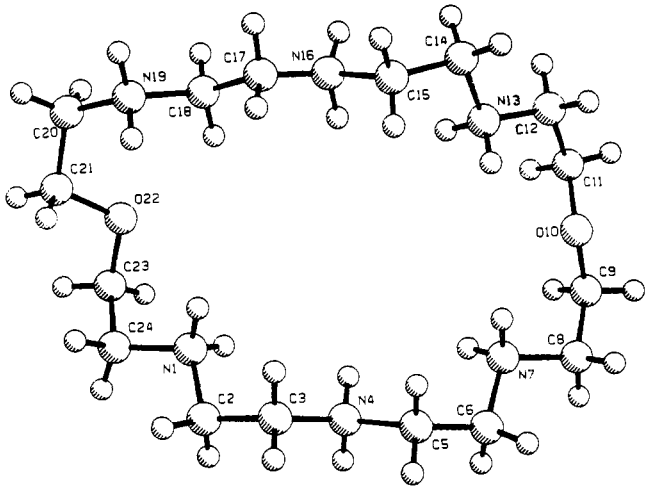


Figure 3. Overhead perspective view of $[24]N_6O_2$ (1).

$k_{cat} = 0.064\ min^{-1}$. Another enzyme analogy to these reactions is that inhibition is observed when an analogue of the substrate such as triphosphate is added.[11] A comparison of the efficiency of 1 with both the uncatalyzed and ATPase-catalyzed dephosphorylation of ATP is shown in Table II.

$$\text{ATP} + 1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 1\cdot\text{ATP} \xrightarrow{k_2} 2 \xrightarrow[-\text{ADP}]{k_3} 1 + P_i \quad (1)$$

$$k_{cat} = (k_2k_3)/(k_2 + k_3) \quad (2)$$

$$\text{Vel} = (k_{cat}[1][\text{ATP}])/(K_m + [\text{ATP}]) \quad (3)$$

In an effort to understand the mode by which ATP interacts with 1, modeling studies were performed utilizing the crystal structure recently obtained for the hexahydrochloride salt of 1[12] and those previously reported for ATP[13] and ADP.[14] The structure of the macrocycle is of interest due to the cupped nature of the ring (Figures 3 and 4). Especially of note are the orientations of the central amines (N4 and N16), which are pointed "down" so that they are almost in line with the two neighboring ammonium moieties. The two ether oxygens lie at the vertices of the long axis of the ellipse, which results in a geometry in which the amines are almost "lined up" along the curve of the long axis. Docking studies have been performed in which both ATP and ADP were inserted into 1 in eight different orientations, lying either along the long axis of the ellipse or perpendicular to it on both the "top" and "underneath" sides of the macrocycle. After the respective substrate was manually docked into the cavity, the receptor–substrate complex was fully minimized to a root mean square of less than 0.01 without constraint, using force-field parameters developed for charged

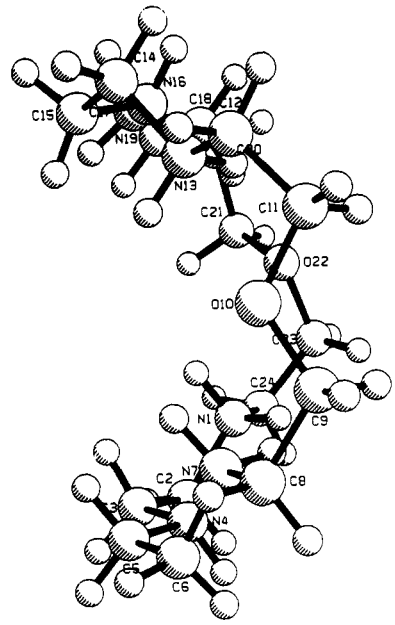
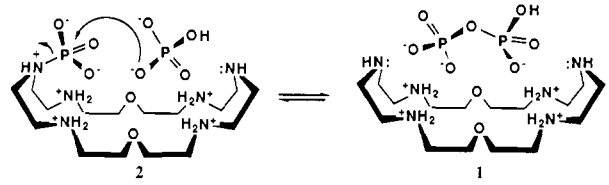


Figure 4. Side view of $[24]N_6O_2$ (1).

Scheme II



molecules such as DNA and polypeptides (AMBER).[15] Energy minimizations using MM2 force-field parameters gave similar results.[16] In all cases after the energy minimization, only the complexes (with either ATP or ADP) that had the terminal phosphate positioned closely (approximately 3.2 Å) to the central nitrogen of the triamine chain achieved significantly lower energy (Figure 5). Hence, this relationship would predict the observed covalent catalysis in the reaction pathway. Also observed is that the oxygens of the two terminal phosphates minimize to lie approximately 1.7 Å away from ammonium protons, indicating the potential for strong hydrogen-bonding interactions.

Kinase Activity

In continuation of the evolution of 1 as an enzyme mimic is the noted effect of the presence of certain metal ions on the reaction. Magnesium ion is required for every ATPase known, and calcium is also implicated in the Mg^{2+},Ca^{2+}-dependent ATPases found in the cytoplasmic membrane of bacteria and the sarcoplasmic membrane of muscle cells.[7] When either of these metal ions is present in the solution with ATP and 1, the resonance in the ^{31}P NMR spectrum due to the phosphoramidate is considerably enhanced and appears before any inorganic phosphate is evident.[17] Comparison of the rates of hydrolysis of the phosphoramidate 2 in the presence and absence of metal ions

(12) Wang, B.; Deffo, T.; Mertes, K. B., submitted for publication.
(13) Larson, A. C. *Acta Crystallogr., Sect. B* **1978**, *34*, 3601–3604.
(14) Shakked, Z.; Viswamitra, M. A.; Kennard, O. *Biochemistry* **1980**, *19*, 2567.
(15) Weiner, S. J.; Kollman, P. A.; Case, D. A.; Singh, C.; Ghio, C.; Alagona, G.; Profeta, S., Jr.; Weiner, P. *J. Am. Chem. Soc.* **1984**, *106*, 765–784.
(16) Thi, J. C.; Allinger, N. L. *J. Am. Chem. Soc.* **1988**, *110*, 2050–2055.
(17) (a) Yohannes, P. G.; Mertes, M. P.; Mertes, K. B. *J. Am. Chem. Soc.* **1985**, *107*, 8288–8289. (b) Yohannes, P. G.; Plute, K. E.; Mertes, M. P.; Mertes, K. B. *Inorg. Chem.* **1987**, *26*, 1751–1755.

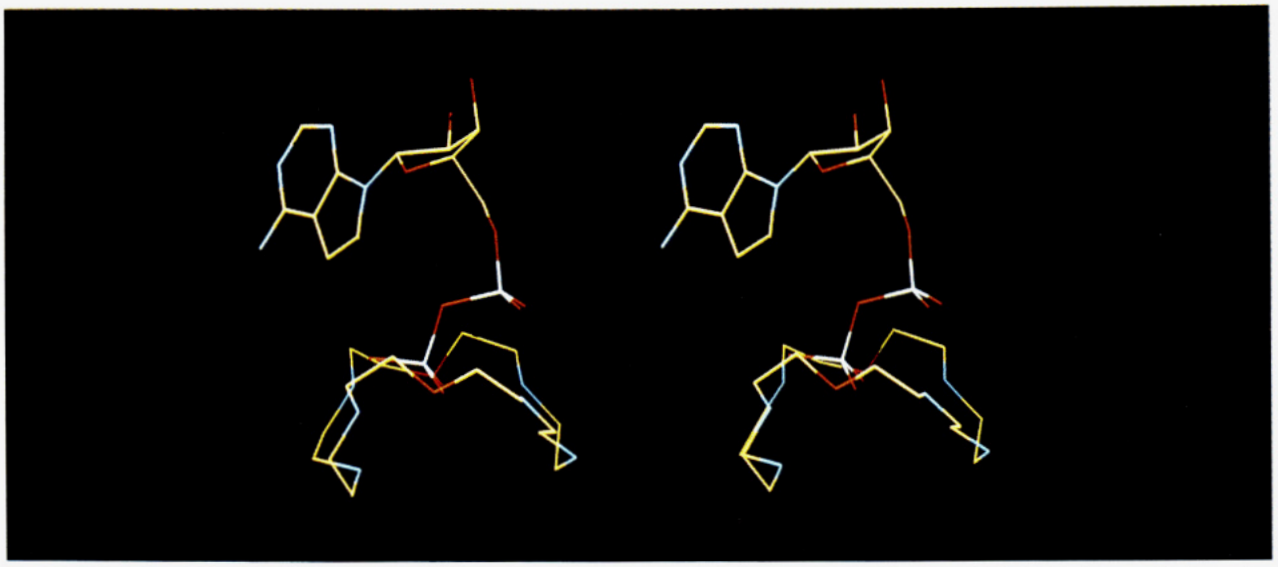


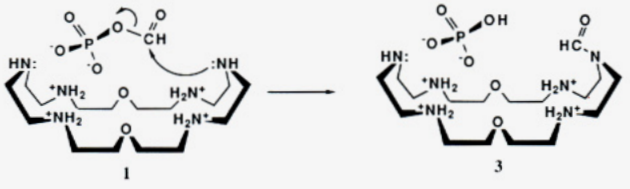
Figure 5. Stereoview photograph of the model for docking of ADP into the macrocyclic cavity of [24]N_6O_2 (1). Atom coloring scheme: carbon, yellow; nitrogen, blue; oxygen, red; phosphorus, white.

indicates that magnesium(II) and calcium(II) tend to stabilize the phosphoramidate. Furthermore, calcium(II) also doubles the rate of dephosphorylation compared to the metal-free catalysis. This may be explained by the fact that ATP potentially chelates to the calcium ion, which may make the nucleotide more compact for approaching the macrocyclic cavity. Of greatest import, however, is the observation of a new resonance signal at −6.22 ppm, which is indicative of pyrophosphate, PP_i, formation. This is the result of retardation in the k_3 step which helps to allow for nucleophilic attack of inorganic phosphate present in solution on the macrocyclic phosphoramidate (Scheme II, Figure 6). There is no evidence of α–β cleavage of the ATP, which could also result in the observation of pyrophosphate. The formation of the new product, pyrophosphate, in the presence of these metals can be classified as allosteric control or "regulation", an additional feature of enzymatic reactions. The activation or deactivation of an enzyme and the modification of the chemistry of an enzyme–substrate complex to produce an alternate reaction pathway are models of allosteric control.[18]

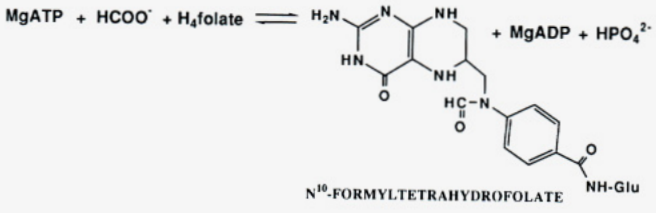
The chemistry observed in the presence of metal ions, namely, phosphorylation of inorganic phosphate, can be classified as a kinase (phosphoryl transfer) reaction. Kinase activity plays a crucial role in a multitude of biochemical transformations[7] and so represents an important class of reactions to model with synthetic catalysts. In order to expand the potential kinase behavior and to investigate the breadth of the substrates recognized by the receptor macrocycle 1, the dephosphorylation of both acetyl phosphate[19] and formyl phosphate[20] has been examined. While acetyl phosphate is readily hydrolyzed by 1 in the same manner as ATP, i.e., the formation of an intermediate phosphoramidate,[19] a different mode of dephosphorylation occurs for formyl phosphate in the presence of macrocycle.

(18) (a) Berridge, M. J.; Irvine, R. F. *Nature (London)* **1984**, *312*, 315–321. (b) Schramm, M.; Selinger, Z. *Science* **1984**, *225*, 1350–1356.
(19) (a) Hosseini, M. W.; Lehn, J.-M. *J. Chem. Soc., Chem. Commun.* **1985**, 1155–1157. (b) Hosseini, M. W.; Lehn J.-M. *J. Am. Chem. Soc.* **1987**, *109*, 7047–7058.
(20) Jiang, Z.; Chalabi, P.; Mertes, K. B.; Jahansouz, H.; Himes, R. H.; Mertes, M. P. *Bioorg. Chem.* **1989**, *17*, 313–329.

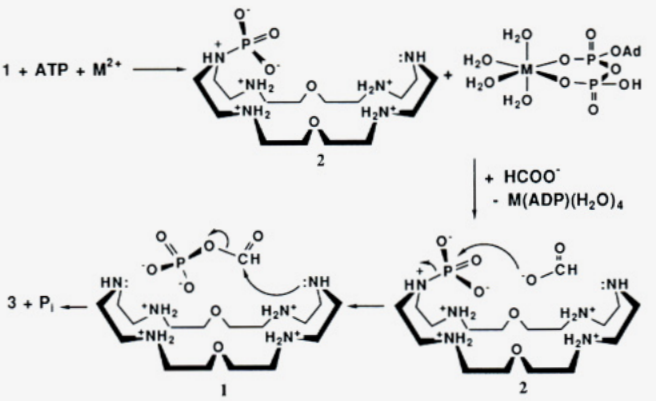
Scheme III



Scheme IV



Scheme V



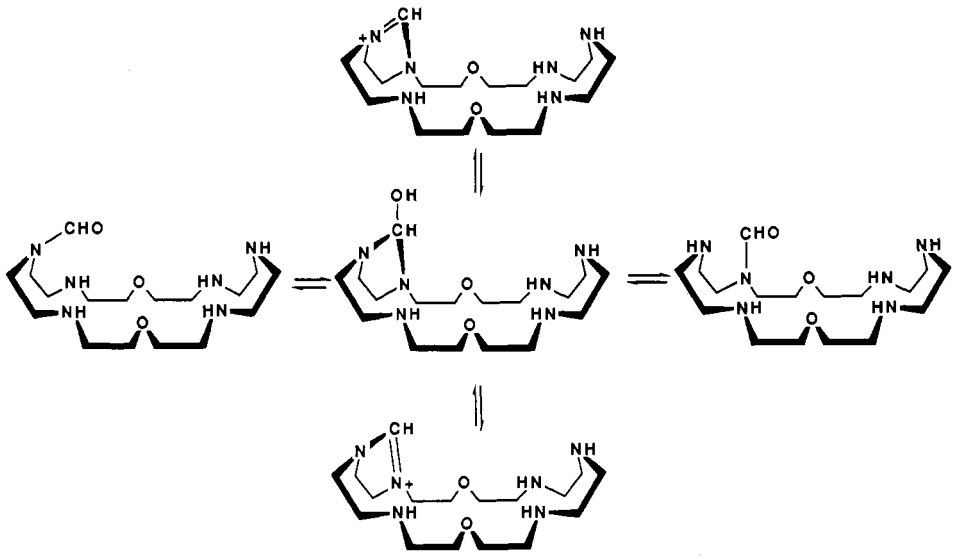
Rather than attack on the phosphoryl group, the amine reacts at the carbonyl site to given an *N*-formyl species, which is stable to further hydrolysis (Scheme III).[21]

N^{10}-Formyltetrahydrofolate Synthetase Mimicry

Formyl phosphate is the purported intermediate in the reaction in which formate is brought into the one-

(21) (a) Smithers, G. W.; Jahansouz, H.; Kofron, J. L.; Himes, R. H.; Reed, G. H. *Biochemistry* **1987**, *26*, 3943–3948. (b) Mejillano, M. R.; Jahansouz, H.; Himes, R. H. *Fed. Proc.* **1987**, *46*, 2225.

Scheme VI



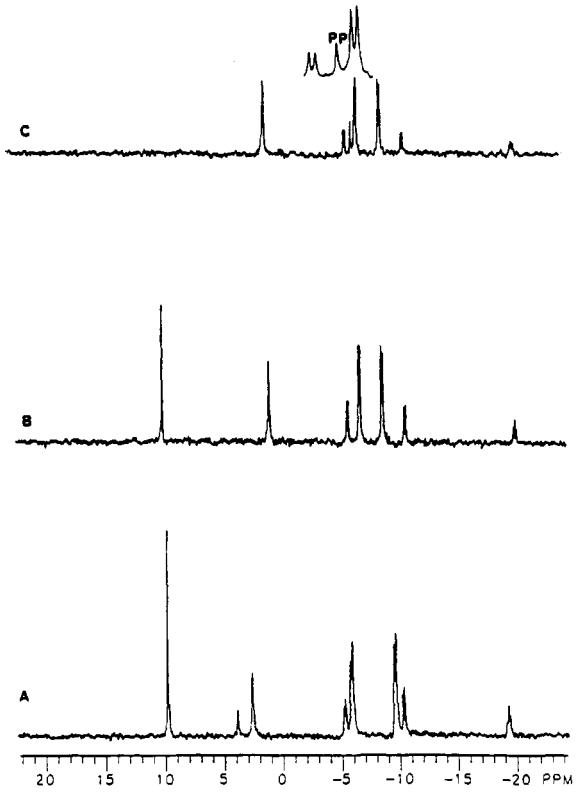


Figure 6. ^{31}P NMR spectra of 0.010 M ATP, 0.015 M [24]N_6O_2 (1), and 0.015 M $CaBr_2$ in 10% D_2O/H_2O at pH 4.5 and 22 °C: (A) at 22 °C with pH adjusted to 4.5 after partial hydrolysis for 38 min at pH 7.6 and 70 °C; (B) after 24 h at pH 4.5 and 22 °C. The chemical shift assignments: ATP, α = −10.7, β = −20.1, γ = −5.7; ADP, α = −8.7, β = −6.7; pyrophosphate, −6.2; orthophosphate, +1.0; phosphoramidate, +10.1 ppm. The chemical shifts are relative to 0 ppm for 85% H_3PO_4 as an external standard (+, downfield).

carbon metabolic pool, wherein formate is activated in the presence of ATP, magnesium(II), and the enzyme N^{10}-formyltetrahydrofolate synthetase. In this reaction the N10 of tetrahydrofolic acid is formylated (Scheme IV).[7] This product is vital for the formylation of methionine to initiate protein synthesis and also in purine biosynthesis. The key requirement for ATP in the reaction suggests that formyl phosphate may be the "missing" ingredient. Although formyl phosphate has not been detected in the enzymatic reaction, two recent findings provide strong evidence of its existence. Synthetic formyl phosphate has been found to phosphorylate ADP and formylate tetrahydrofolate when the enzyme is present.[21] More recently it has been found that the enzyme catalyzes the formation of formyl phosphate from ATP and formate.[22]

The discovery of the different mode of attack of 1 on formyl phosphate, compared to the other dephosphorylations, provided the key to expand the catalytic capabilities of the macrocycle to a multistep enzyme pathway: the activation of formate as in N^{10}-formyltetrahydrofolate synthetase. The success of such an undertaking rested on the observed kinase activity promoted by the macrocycle in pyrophosphate synthesis, as well as on the mode of dephosphorylation of formyl phosphate (carbonyl attack). When ATP, magnesium(II), formate, and 1 are placed in aqueous solution at pH 7, not only does the initial formation of the phosphorylated macrocycle occur as anticipated, but the *N*-formyl macrocyclic product, **3**, also forms as verified by ^{13}C NMR (Scheme V).[23] Both magnesium(II) and calcium(II) catalyze the activation, and in the absence of ATP the reaction does not occur. As in the case of the actual enzyme, the proposed intermediate formyl phosphate is not detected, but when the reaction is done in 97% ^{18}O-labeled water, 25% unlabeled phosphate is found. This can only result from attack of the phosphoramidate by the unlabeled oxygen on formate and provides strong evidence that a fleeting intermediate formyl phosphate species is involved. Another unanticipated result is the incorporation of ^{18}O into the amide oxygen of **3**. This is proposed to occur as shown in Scheme VI and models the N5–N10 formyl transfer in tetrahydrofolic acid which also occurs in biological sequences.

Conclusions

The analogies between the simple monomacrocyclic polyaza molecule and phosphoryl-transfer enzymes are shown in Table III. As seen from Table III, a number

(22) Mejillano, M. R.; Jahansouz, H.; Matsunaga, T. S.; Kenyon, G. L.; Himes, R. H. *Biochemistry* **1989**, *28*, 5136–5145.
(23) Jahansouz, H.; Jiang, Z.; Himes, R. H.; Mertes, M. P.; Mertes, K. B. *J. Am. Chem. Soc.* **1989**, *111*, 1409–1413.

Table III
Analogies of [24]N_6O_2 to Enzymes

1. Reactions proceed in neutral aqueous media.
2. Anionic binding occurs via electrostatic and hydrogen-bonding interactions.
3. K_m values approximate those of the ATPases.
4. Metal-ion catalysis and regulation are observed.
5. The reaction is dependent on a Michaelis-like complex.
6. Nucleophilic catalysis provides an intermediate, unstable, phosphorylated catalyst.
7. The phosphorylated intermediate **2** reacts with water (as in ATPases) or a second substrate (carboxylate or phosphate, as in kinases).
8. Competitive inhibition occurs when substrate analogues are used.

of enzyme characteristics are also inherent in the macrocyclic analogue. Nonetheless, despite the chemical similarities, there is still a wide gap between hydrolysis rates for the enzyme and **1** (Table II). Given that the molecule is so simple, it should be possible, by appropriate design, to improve the rates, once the intricacies of ring size and heteroatom placement are better understood. However, given the eons of evolution and complexities inherent in enzymes, it is improbable that the simplistic approach of biomimetic chemistry will match the efficiency of nature.

The important contributions of collaborators Jean-Marie Lehn and Mir Wais Hosseini at Université Louis Pasteur, Richard H. Himes at the University of Kansas, Piero Paoletti and Antonio Bianchi at the University of Florence, Enrique Garcia-España at the University of Valencia, and all of the researchers on this project are gratefully acknowledged. Special thanks go to Daryle Busch for suggesting the article and Doug Rees and Barbara Hsu for their assistance with the color graphics. This work was supported by National Institutes of Health Grant GM33922.

Registry No. 1, 43090-52-4; phosphotransferase, 9031-09-8.