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New nucleotide analogues and their applications in AIDS research and other biological systems

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New Nucleotide Analogues and Their Applications
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Qi-Feng Ma

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To my wife, Rong Guo,
and my son, Jun Ma,
for their love, support and encouragement.

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I wish to acknowledge Dr. George L. Kenyon for his help, encouragement and excellent advice throughout my graduate study. His supervision not only has led to the completion of my research project, but also has deeply influenced my future scientific career.

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New Nucleotide Analogues and Their Applications in AIDS Research and Other Biological Systems

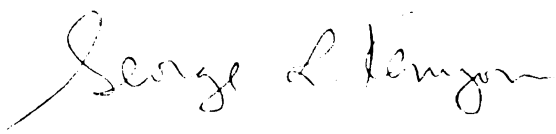
Qi-Feng Ma

Abstract

A new class of nucleotide analogues, nucleoside 5'-[α,β-imido] and 5'-[α,β-N-methylimido]triphosphates, have been synthesized and studied in several enzyme systems. Thymidine triphosphate analogues, such as thymidine 5'-[α,β-imido]triphosphate (TMPNPP), 3'-azido-3'-deoxythymidine 5'-[α,β-imido]triphosphate (AZTMPNPP), 3'-azido-3'-deoxythymidine 5'-[β,γ-imido]triphosphate (AZTMPPNP), thymidine 5'-[α,β:β,γ-diimido]triphosphate (TMPNPNP), and dideoxythymidine 5'-[α,β-imido]triphosphate (ddTMPNPP), have proved to be good competitive inhibitors of human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT). They have also been used to study the structure-activity relationship of nucleoside analogue inhibitors. These studies have led to a finding that the reported inhibitory potency of AZTTP for HIV-1 RT is an artifact due to the use of homopolynucleotides as templates.

Adenosine triphosphate analogues have been tested in ATP-utilizing enzyme reactions. Adenosine 5'-[α,β-imido]diphosphate (AMPNP) and adenosine 5'-[α,β-N-methylimido]diphosphate (AMPN(Me)P) are substrates for creatine kinase and pyruvate kinase; adenosine 5'-[α,β-imido]triphosphate (AMPNPP) and adenosine 5'-[α,β-N-methylimido]triphosphate (AMPN(Me)PP) are substrates for hexokinase as well as creatine

kinase. AMPNPP, adenosine 5'-[α,β:β,γ-diimido]triphosphate (AMPNPNP), and adenosine 5'-[α,β:α,β'-diimido]triphosphate ($AMP(NP)_2$) are good inhibitors for S-adenosylmethionine synthetase with AMPNPP as the most potent substrate analogue inhibitor for the enzyme. AMPNPNP turned out to be an active site titrant for S-adenosylmethionine synthetase and has potential usefulness in future study. These studies indicate that α,β-imido analogues of NTP's are useful in biological studies and should become as important as their corresponding β,γ-imido analogues, adenosine 5'-[β,γ-imido]triphosphate (AMPPNP) and guanosine 5'-[β,γ-imido]triphosphate (GMPPNP).



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List of Abbreviations

$AG^{R}MP$-1	analytical grade macroporous anion resin
AMPCPP	adenosine 5'-[α,β-methylene]triphosphate
AMPPCP	adenosine 5'-[β,γ-methylene]triphosphate
AMPCPCP	adenosine 5'-[α,β:β,γ-dimethylene]triphosphate
AMPCP	adenosine 5'-[α,β-methylene]diphosphate
AMPNPNP	adenosine 5'-[α,β:β,γ-diimido]triphosphate
AMPNPP	adenosine 5'-[α,β-imido]triphosphate
AMPPNP	adenylyl imidodiphosphate
AMPNP	adenosine 5'-[α,β-imido]diphosphate
$AMP(NP)_2$	adenosine 5'-[α,β:α,β'-diimido]triphosphate
AMPN(Me)P	adenosine 5'-[α,β-N-methylimido]diphosphate
AMPN(Me)PP	adenosine 5'-[α,β-N-methylimido]triphosphate
AMPPN(Me)P	adenosine 5'-[β,γ-N-methylimido]triphosphate
AZT	3'-azido-3'-deoxythymidine
AZTMPNP	3'-azido-3'-deoxythymidine 5'-[α,β-imido]diphosphate
AZTMPNPP	3'-azido-3'-deoxythymidine 5'-[α,β-imido]triphosphate
AZTTP	3'-azido-3'-deoxythymidine triphosphate
AZTMPPNP	3'-azido-3'-deoxythymidine 5'-[β,γ-imido]triphosphate
ddTMPNP	dideoxythymidine 5'-[α,β-imido]diphosphate
ddTMPNPP	dideoxythymidine 5'-[α,β-imido]triphosphate
DE	diethylaminoethyl cellulose

DMF	dimethylformamide
EDTA	ethylenediaminetetraacetic acid
HIV-1 RT	human immunodeficiency virus-1 reverse transcriptase.
LSIMS	liquid secondary ion mass spectrometry
m-CPBA	*meta*-chloroperoxybenzoic acid
NMPNPNP	nucleoside 5'-[α,β:β,γ-diimido]triphosphate
NMPNPP	nucleoside 5'-[α,β-imido]triphosphate
PCPCP	bis[(dihydroxyphosphinyl)methyl]phosphinate
PEI	polyethylenimine
PMSF	phenylmethanesulfonylfluoride
PNP	imidodiphosphate
PNPNP	diimidotriphosphate
PN(Me)P	N-methylimidodiphosphate
Sp	sulfopropyl
YEPD	yeast extract, peptone and dextrose
TEAB	triethylammonium bicarbonate
TMPNP	thymidine 5'-[α,β-imido]diphosphate
TMPNPNP	thymidine 5'-[α,β:β,γ-diimido]triphosphate
TMPNPP	thymidine 5'-[α,β-imido]triphosphate

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Introduction

I. Imidotriphosphate Analogues of Nucleosides (NMPPNP's and NMPNPP's)

Nucleoside triphosphates (NTP's) and 2'-deoxynucleoside triphosphates (dNTP's) are very important in biological systems. They are the building blocks for DNA and RNA and are substrates and allosteric effectors for many enzymes and receptors. Analogues of NTP precursors also consist of a class of important drugs for chemotherapy [e.g., AZT for AIDS (Mitsuya et al., 1985); 5'-fluoro-2'-deoxyuridine for cancer (Prusoff et al., 1979); and acyclovir for herpes infections (Robins et al., 1981; Schaeffer et al., 1958)]. The unique features of NTP's have attracted many scientists to study their interactions with enzymes or receptors, and a class of nonhydrolyzable analogues of NTP's, such as imido or methylene triphosphates of nucleosides, have emerged for these purposes.

A. Chemistry of Imidopolyphosphates

The crystal structure of sodium imidodiphosphate is very similar to the structure of pyrophosphate (Larsen et al., 1969). The bond angle of PNP (127.2°) is similar to that of POP (128.7°) and the bond distance of P-N (1.68 Å) is nearly identical to that of P-O bond (1.63 Å). Compared with the PCP bond, the PNP bond is closer to the POP bond (see Fig. 1-1) (Yount et al., 1971; Lovell et al., 1964).

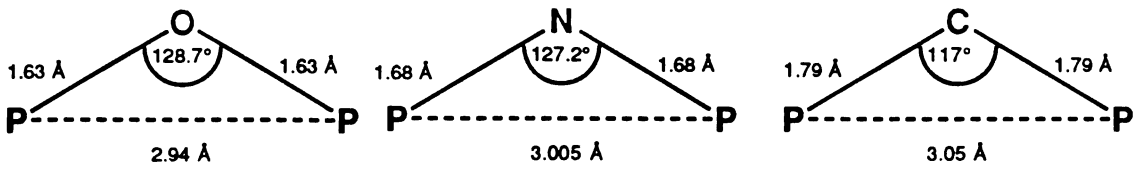


Fig. 1-1. Comparison of the P-X-P bond angles and P-X bond distances

In general, imido links are less stable than oxygen links under weakly acidic conditions, but are equally, or more stable, in basic solutions (Quimby et al., 1960). The PNP bond is also more stable than the POP bond in strongly acidic conditions. The stability of PNP bond makes it uniquely appropriate for biochemical studies.

Further studies on PNP bond in solution were carried out by ^{31}P, ^{17}O and ^{15}N NMR. The pK_a values for the tetramethyl and tetraethyl esters of PNP have been measured

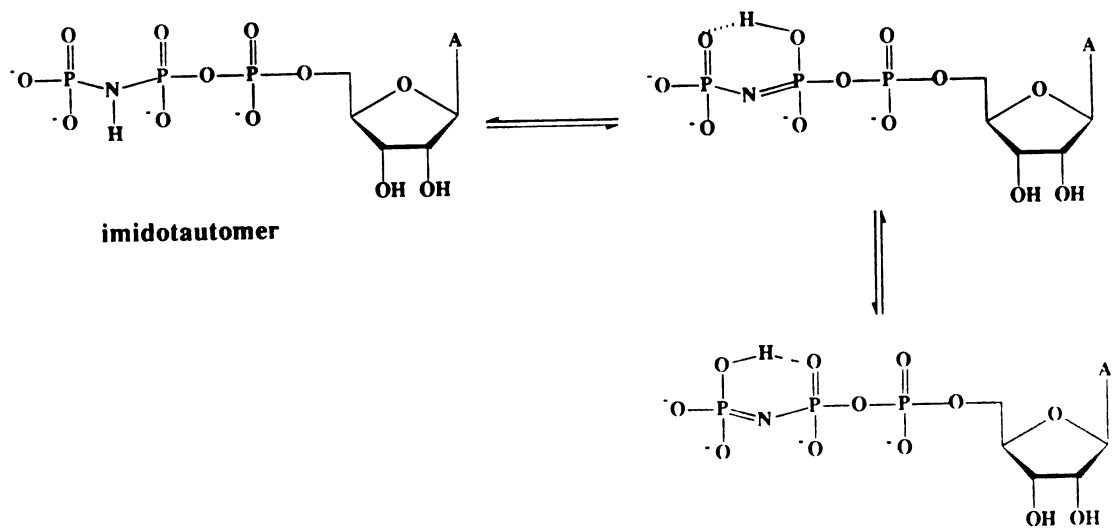


Fig. 1-2. Proposed tautomers of AMPPNP showing internal H bonding (·····)

as 2.6 and 3.7, respectively (Kireev et al., 1970). ^{31}P NMR spectral properties of adenosine 5'-[β,γ-imido]triphosphate (AMPPNP) as a function of both pH and Mg^{++} concentration have been reported, and the titration behavior of AMPPNP is markedly different from that of ATP (Tran-Dinh et al., 1977). Using ^{15}N and ^{17}O NMR, Reynolds et al. (1983) demonstrated that the predominant structure of PNP bond is imido tautomer (Fig. 1-2). These studies further suggested that the PNP bond in solution is basically the same as the crystal structure and is isosteric with POP bond.

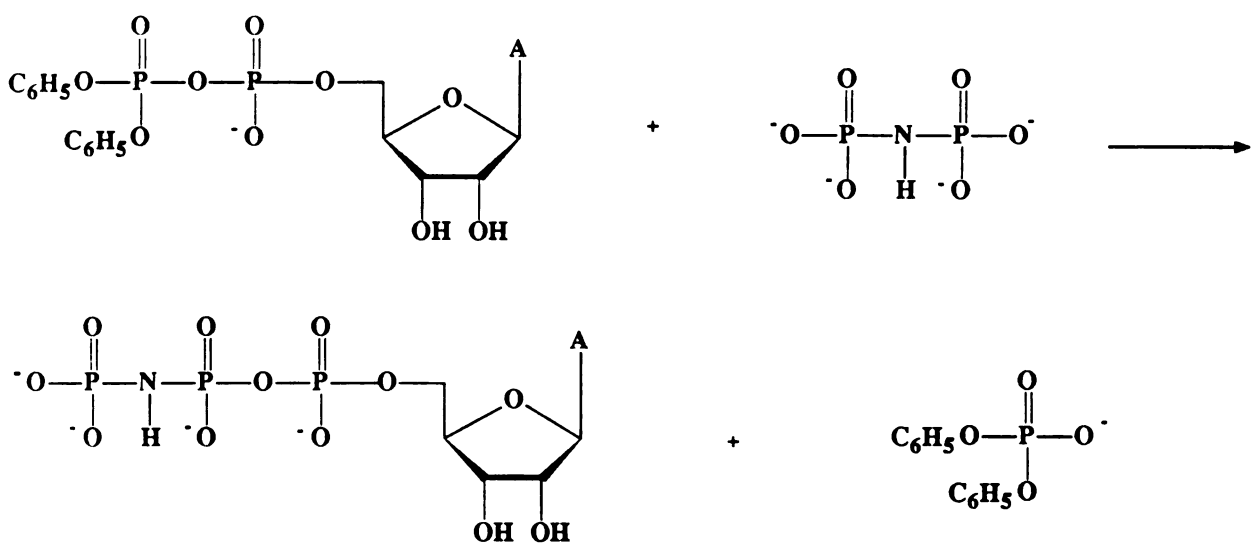
B. Biological Activity of Nucleoside Imidotriphosphates

Because the similarity of the PNP bond to the POP bond, most of the nucleoside imidotriphosphates can bind to the enzymes using NTP's as substrates. With the exception of *E. coli* alkaline phosphatase, the PNP bond is resistant to enzymatic cleavage (Yount et al., 1971).

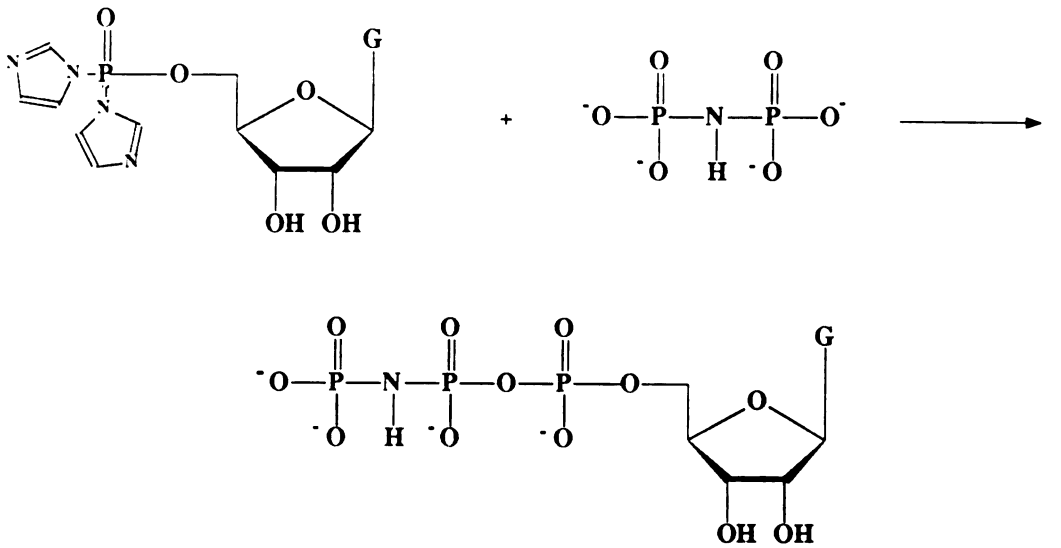
The known imidotriphosphate analogues of NTP are nucleoside 5'-[β,γ-imido]triphosphates, such as adenosine 5'-[β,γ-imido]triphosphate (AMPPNP) and guanosine 5'-[β,γ-imido]triphosphate (GMPPNP). These analogues proved to be useful in biological studies. AMPPNP has been used to verify that the energy of dephosphorylation liberated during the polymerization of actin is not used to faciliate the polymerization (Cooke et al., 1973). AMPPNP has also been used to locate adenylate cyclase cytochemically by lead precipitation of the imidodiphosphate released (Wagner et al., 1972). The function of GTP and GDP in G proteins has also been studied by the nonhydrolyzable GTP analogues (Gomperts, 1983; Breitwieser et al., 1985). Nucleoside imidotriphosphates should be specially useful in locating the active site of NTP-using enzymes by cocrystallization with enzymes.

C. Synthesis of Nucleoside Imidotriphosphates

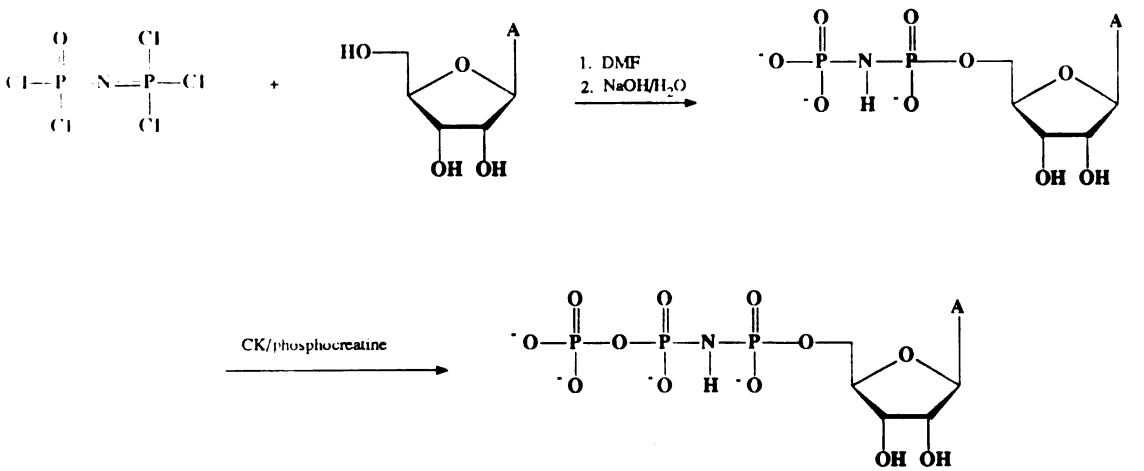
β,γ-Imidotriphosphate analogues of nucleosides have been synthesized using the methods described by Yount (1971) and Eckstein (1971). These syntheses are illustrated in Scheme 1-1 and Scheme 1-2, respectively. No direct methods can be followed to synthesize 5'-[α,β-imido]triphosphates of nucleosides. A proposal was made by Kenyon (1985) to prepare adenosine 5'-[α,β-imido]triphosphate (see Scheme 1-3). The same methods have also been used to synthesize the α,β-imido analogues of NDP at the same time as I was working on synthesizing the [α,β-imido]triphosphate analogues (Tomasz et al., 1988).



Scheme 1-1. Synthesis of AMPPNP



Scheme 1-2. Synthesis of GMPPNP



Scheme 1-3. Synthesis of AMPNPP

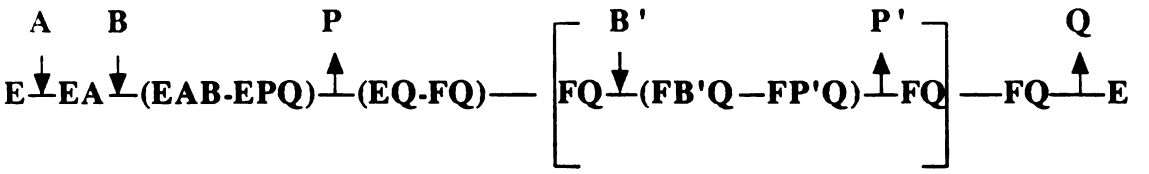
II. HIV-1 Reverse Transcriptase (HIV-1 RT)

A. Structure and Function of the Enzyme

HIV-1 RT is encoded by *pol* gene, a large open reading frame located near the center of HIV-1 genome (Jacks et al., 1988). Native HIV-1 RT is a heterodimer containing 66 and 51 kDa subunits (Lightfoote et al., 1986; di Marzo veronese et al., 1986). The enzyme contains two domains: a DNA polymerase (reverse transcriptase) activity domain and a ribonuclease (RNase) H activity domain (Hansen et al., 1987; Starnes et al., 1989). The polymerase domain is located in the N terminal region while RNase H domain is located in the C terminal region (Tisdale et al., 1988).

The polymerase function of HIV-1 RT has been characterized in great detail and is rather similar to that of other DNA polymerases (Hoffman et al., 1985). The most profound difference is its ability to copy both RNA and DNA. HIV-1 RT is moderately processive using RNA templates but much less so using DNA templates. The synthesis is nearly distributive in DNA templates (Reardon et al., 1990; Hiz et al., 1991).

B. Kinetic Mechanism of HIV-1 RT



F = E: enzyme B = B': nucleoside triphosphate [ ] : Processive events
P = P': PPi A = Q: template-primer

Scheme 1-4. Kinetic mechanism of HIV-1 RT

It was suggested that the reaction pathway for DNA synthesis catalyzed by HIV-1 RT is ordered, with the template-primer and free enzyme combining to form the first complex in the reaction sequence (see Scheme 1-4) (Majumdar et al., 1988).

The first nucleoside monophosphate moiety incorporation is distributive. After that, the enzyme/template-primer complex undergoes a conformational change and the subsequent NMP moiety incorporation is processive. The dissociation of the enzyme from the template-primer is the rate-limiting step (Reardon et al., 1990).

The chemical events are illustrated in Fig.1-3. After the formation of the ternary complex of enzyme-NTP-template-primer, HIV-1 RT catalyzes the cleavage of the α,β-phosphate bond and the formation of the phosphodiester bond. The 3'-OH group in the deoxyribose moiety of dNTP is essential for the synthesis of DNA.

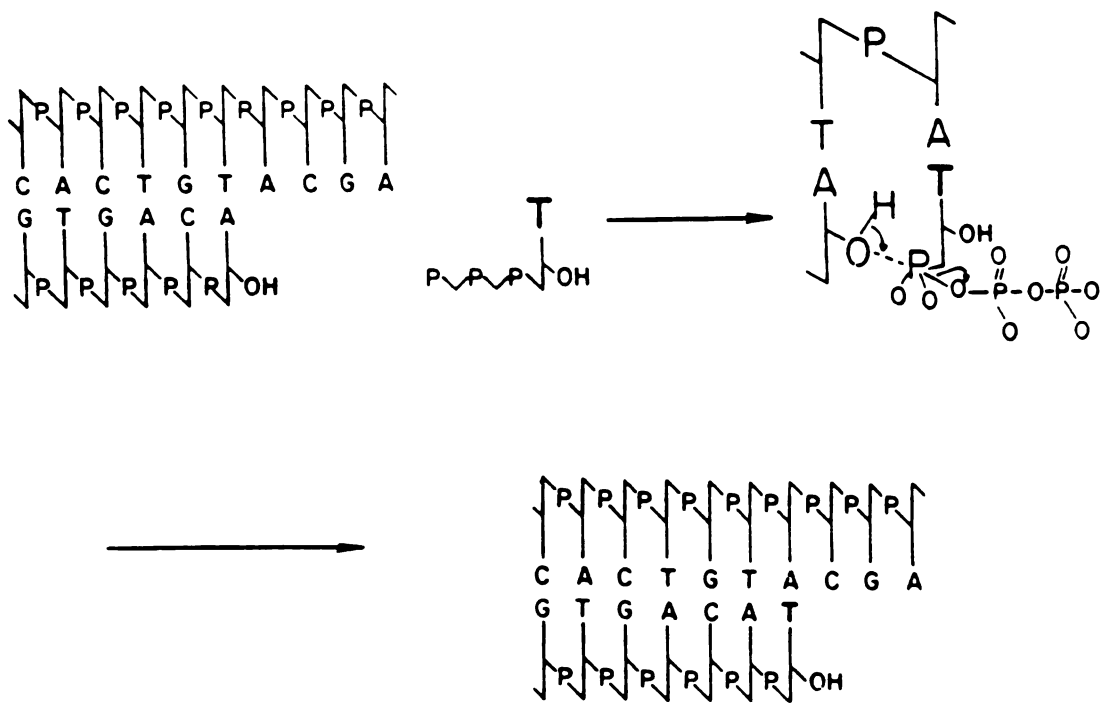


Fig. 1-3. Chemical events of NMP moiety incorporation

C. Roles of HIV-1 RT and Its Inhibitors

HIV-1 RT catalyzes proviral DNA synthesis and plays an important role in HIV-1 life cycle (Haseltine, 1989). Several characteristics of the enzyme make it an important target for the design of anti-AIDS drugs. HIV-1 RT is encoded by the virus. Specific inhibitors for HIV-1 RT will not affect the enzymes of the host. HIV-1 RT catalyzes both DNA synthesis (DNA polymerase activity) and the removal of RNA from RNA-DNA heteroduplex (RNase H activity). Its inhibitors will interfere with several steps in the viral life cycle and make the inhibitors more effective.

The existing HIV-1 RT inhibitors can be classified as nucleoside analogues, pyrophosphate analogues, oligonucleotides and other non-nucleotide inhibitors(Mitsuya et al., 1985; Mitsuya et al., 1986; Sandstrom et al., 1985; Nakane et al., 1990). Most of the nucleoside analogues are DNA chain terminators, such as DDI, DDC and AZT. In vitro studies have shown that these 3'-deoxynucleoside analogues are potent competitive inhibitors for HIV-1 RT. Although these inhibitors have been studied extensively, their inhibition mechanisms are not very clear. For example, AZTTP, the effective form of AZT, has a reported K_i value of 4 nM. This potent inhibition has been attributed to different inhibition mechanisms, such as substrate inhibition (Kedar et al., 1990) and product inhibition (Müller et al., 1991). Further understanding of the inhibition mechanism is important for the discovery of new anti-AIDS drugs.

III. Goals of the Project

The major goal of my project was to design inhibitors for HIV-1 RT and to study the mechanism of HIV-1 RT inhibition by nucleotide analogues. As discussed above, HIV-1 RT catalyzes the incorporation of an NMP moiety into the growing DNA chain by cleaving the α,β-phosphate linkage. If the bridging oxygen between α,β-phosphate moieties of NTP is replaced by another atom, which makes the bond uncleavable, a competitive inhibitor of HIV-1 RT would be generated. Since a PNP bond is the closest analogue of the POP bond, and resistant to enzymatic cleavage, α,β-imidotriphosphate analogues of nucleosides have been designed as inhibitors of this type. One purpose of studying these inhibitors is to examine the substitutive effects of NTP on the affinity to HIV-1 RT. The results should be useful for the discovery of new HIV-1 RT inhibitors. We can also use these analogues to study the inhibition mechanisms of existing 3'-deoxynucleoside analogues for HIV-1 RT. Furthermore, this new class of compouds has the potential for AIDS therapy if the problem for their delivery is solved.

Since nucleside 5'-[α,β-imido]triphosphates have never been made before, the first goal in my project was to develop synthetic methods for these analogues. Obviously, the synthetic pathways for nucleoside 5'-[β,γ-imido]triphosphates cannot be followed to synthesize 5'-[α,β-imido]triphosphate analogues.

As a new class of compounds, we needed to investigate their more general biochemical usefulness. Several enzymes have been chosen for this purpose, and the results show that 5'-[α,β-imido]triphosphate analogues of nucleosides should become as important as the corresponding β,γ-imido analogues.

2

Syntheses , Characterizations and Biological Activities of Imido and Methylimido Analogs of ATP and ADP

I. Abstract

N-methylimidodiphosphate (PN(Me)P) has been synthesized by hydrolysis of N-methylimidodiphosphoryl tetrachloride. PN(Me)P was coupled with 5'-tosyladenosine to produce adenosine 5'-[α,β-N-methylimido]diphosphate [AMPN(Me)P] which in turn was treated with phosphocreatine in the creatine kinase reaction to generate adenosine 5'-[α,β-N-methylimido]triphosphate [AMPN(Me)PP]. Using the Michelson synthesis, PN(Me)P was coupled with adenosine 5'-monophosphate to produce adenylyl N-methylimidodiphosphate [AMPPN(Me)P]. Adenosine 5'-[α,β-imido]diphosphate [AMPNP] was synthesized by the reaction of adenosine with dichlorophosphoryl trichloroiminophosphorane, and adenosine 5'-[α,β-imido]triphosphate [AMPNPP] was prepared from AMPNP in the same way as AMPN(Me)PP synthesis. All five new purine nucleotide analogs were characterized by mass spectrometry and ^{31}P NMR spectroscopy. Their enzyme kinetic parameters (V_{max} and K_m values) were measured in the creatine kinase reaction and directly compared to similar kinetic parameters obtained for ADP and ATP. AMPN(Me)P was shown to have a V_{max} value ca. 40% of that of ADP although its Km value is ca. 100 times greater than that for ADP. AMPNP turned to be a better substrate than AMPN(Me)P with a K_m value 4 times lower than AMPN(Me)P. AMPN(Me)PP and AMPNPP were also substrates, whereas AMPPN(Me)P showed no detectable inhibitory properties.

II. Introduction

In 1971, Yount and co-workers (1971) first reported the synthesis and inhibitory properties of adenylyl imidodiphosphate (AMPPNP), the β,γ-N-H analog of ATP. Since that time, AMPPNP has been widely used in enzymological studies by virtue of its close structural similarity to ATP.

Since the P-N-P bridge in AMPPNP is relatively inert to hydrolysis both in basic solution and in the presence of enzymes that catalyze either the hydrolysis or transfer of the γ-phosphoryl group of ATP, this compound has been a useful competitive inhibitor of both kinases and ATPases. In a few cases, P-N-P bonds can be cleaved by certain phosphoryl transfer enzymes (Yount et al., 1971; Taylor, 1981; Harmony et al., 1983). Although the α,β-methylene analogs (AMPCPP and AMPCP) have been synthesized and biologically studied, their corresponding imido analogs have not been reported. Since the α,β-methylene analogs have had such a mixed record of success in binding productively to the ADP and ATP sites of enzymes (Cuee et al., 1988; Morley et al., 1970; Burger et al., 1970; Taketa et al., 1971; Krug et al., 1973; Cooke et al., 1973; Ashman et al., 1975; Horak et al., 1976; Mannerz et al., 1976; Rose et al., 1979; Milner -White et al., 1983), we were interested in seeking some analogs which have higher affinity to these enzymes. α,β-Imido analogs of nucleosides were our primary choice. We reasoned that the N-H analogs should be better accepted because (1) the bridging N-H still has a lone pair of electrons and can therefore act as either a hydrogen bonding acceptor or coordinate to an electrophilic metal ion and (2) crystal structures for imidodiphosphate (PNP) and pyrophosphate show that bond lengths and bond angles of the P-N-P and P-O-P linkages are nearly identical (Larsen et al., 1969).

In this chapter, we present the first syntheses of the corresponding N-methyl and N-H analogs, namely adenosine 5'-[α,β-N-methylimido]diphosphate (AMPN(Me)P),

adenosine 5'-[α,β-N-methylimido]triphosphate (AMPN(Me)PP), adenylyl N-methylimidodiphosphate (AMPPN(Me)P), adenosine 5'-[α,β-imido]diphosphate (AMPNP), and adenosine 5'-[α,β-imido]triphosphate (AMPNPP). It is hoped that these new analogs can be used to study the interactions between enzymes and substrates and to probe for bulk tolerance in ADP- and ATP- requiring enzymatic systems, especially among isoenzymes. They may also serve as competitive inhibitors.

We also report their full characterizations using ^{31}P NMR, liquid secondary ion mass spectrometry (LSIMS) and pH-titration curves. Finally, some kinetic studies have been performed for these analogs in the rabbit muscle creatine kinase reaction. The results are compared with corresponding kinetic parameters for ADP and ATP under the same reaction conditions.

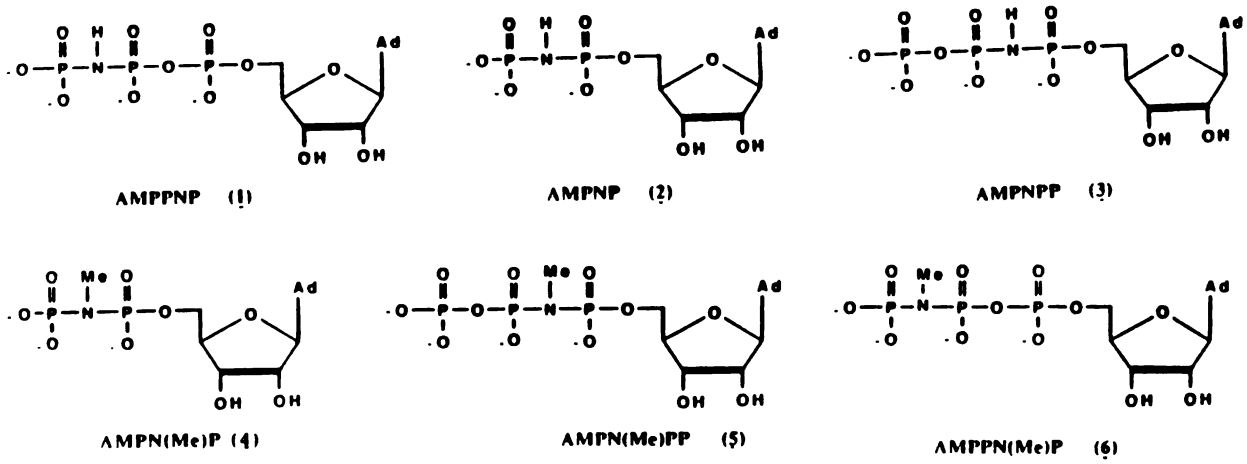


Fig. 2-1 Structures of imido and methylimido analogs of ATP

III. Experimental

A. Materials and Methods

Adenosine 5'-monophosphoric acid (AMP), phosphocreatine, glucose, creatine kinase, glucose-6-phosphate dehydrogenase, pyruvate kinase, hexokinase, PEP, NADH, NADP, ADP, and ATP were all purchased from Sigma Chemical Co. Diethyl chlorothiophosphate, diphenyl chlorophosphate, 5'-tosyladenosine, and trimethylsilyl iodide were all products of Aldrich Chemical Co. Pyridine was heated at reflux over NaOH pellets, distilled, and stored over molecular sieves (Aldrich, type 4A, 4-8 mesh). CH_2Cl_2 (Aldrich) was heated at reflux over CaH_2, distilled and stored over molecular sieves. Dimethylformamide (Aldrich) was dried over molecular sieves for more than 2 weeks. Trioctylamine, tributylamine, and triethylamine (Aldrich) were all distilled prior to use and stored at 4-5°C. Triethylammonium bicarbonate solutions (1 M) were prepared by bubbling CO_2 through a sintered glass diffuser into a triethylamine solution until the pH reached 8.5. DEAE and QAE Sephadex A-25 (Pharmacia) were converted to their HCO_3^- form with 1 M solutions of $NaHCO_3$. Other chemicals were all obtained from either Aldrich or J. T. Baker and used directly.

High-performance liquid chromatography was performed on a Beckman 110A instrument using an ODS column (partisil M9 10/50 ODS-3, Whatman) with a linear gradient in solvent B (0.1 M TEAB/EtOH) of 0.25%/min as eluent (Mahoney et al., 1984). The chromatographic fractions were detected either by uv at 275 nm or by use of polyethylenimine-cellulose thin-layer chromatography (PEI-cellulose TLC) (Rowley et al., 1974). The total amount of phosphorus in pooled fractions was measured by acid-labile phosphate method (Ames et al., 1966). ^{31}P NMR spectra were recorded either at 40.5 MHz using a Varian XL-100 Spectrometer or at 79.5 MHz using a Nicolet NTCFT-1180

spectrometer. Broadband proton decoupling was routinely employed. a sweep-width of 4000 Hz, a probe temprature of 22°C, and broadband ^{1}H decoupling were used in ^{31}P NMR measurements, and chemical shifts were determined relative to 85% H_3PO_4 with positive shifts being downfield of the reference. ^{1}H NMR spectra were taken at 80 MHz on a Varian FT-80 spectrometer. High-resolution LSIMS spectra were recorded at the University of California (San Francisco) on a Kratos MS-50 mass spectrometer (negative ion probe). pH-titration curves were generated and analyzed as described previously (Gerlt et al., 1983; Reynolds et al., 1983).

B. Creatine Kinase Kinetic Studies

Creatine kinase assays were performed and computer analyzed essentially as described by Cook et al. (1981). In the forward reaction (ATP consumption) the assay solutions (pH 9.0, 25°C) contained glycine (0.2 M), $Mg(OAc)_2$ (12 mM), KOAc (0.2 M), creatine (66 mM), PEP (2.8 mM), NADH (0.23 mM), lactic dehydrogenase (80 U/ml), pyruvate kinase (65 U/ml), creatine kinase (23 U/ml), and varying concentrations of ATP (or ATP analogs). In the reverse reaction (ATP formation, pH 7, 25°C) the assay solutions contained glycine (0.2 M), $Mg(OAc)_2$, (12 M), glucose (71 mM), phosphocreatine (29.5 mM), NADP (0.94 mM), hexokinase (30 U/ml), glucose-6-phosphate dehydrogenase (33 U/ml), creatine kinase (12 U/ml), and varying concentrations of ADP (or ADP analogs). Initial rates were measured by the change of ultraviolet absorbance at 340 nm.

Progress in the creatine kinase reactions could also be followed semi-quantitatively using the PEI-cellulose TLC method of Rowley and Kenyon (1974).

C. Synthesis

a. Preparation of PN(Me)P

N-Methylimidodiphosphate [PN(Me)P] was synthesized by the methods of Reynolds et al. (1983) and was used as a precursor for the synthesis of N-methylimido analogs of nucleotides.

(1) N-Methylimidodiphosphoryl tetrachloride . CH_2Cl_2 was dried by heating at reflux over CaH_2. Dichlorophosphoryl trichloroiminophosphorane (Emsley et al., 1971) (15.00 g, 55.7 mmol) was dissolved in 50 ml of dry CH_2Cl_2 in a 200-ml round bottom flask equipped with a magnetic stir bar, addition funnel and $CaCl_2$ drying tube. The reaction vessel was cooled to ca. -20°C using a dry ice/acetone bath. Next, absolute methanol (1.785 g, 55.7 mmol), dissolved initially in 25 ml of dry CH_2Cl_2, was added dropwise with stirring using the addition funnel. Stirring was continued at -20°C for 30 min and then at room temperature for 2 h. The solvent was removed under reduced pressure, and the last traces of HCl were removed by application of high vacuum (0.1 Torr) at room temperature. The resulting oil was then heated at 65-70°C in an oil bath for 4 h, while being protected from moisture with a $CaCl_2$ drying tube. The product was purified by vacuum distillation at 72-73°C (0.01 Torr) [literature bp 95-98 °C, 0.3 Torr (Riesel et al., 1977)] This material rapidly solidified to give a white glassy solid, mp 49-50°C [literature mp 50-51°C (Riesel et al., 1977)] which weighed 8.78 g (59.5% of the theoretical amount). ^1H NMR ($CDCl_3$): δ 3.38 (t, J_{PN} = 13.6 Hz). Anal. Calcd for $C_1H_3NO_2P_2Cl_4$: C, 4.54; H, 1.14; N, 5.29; P, 23.39. Found: C, 4.40; H, 1.16; N, 5.14; P, 23.18.

(2) N-Methylimidodiphosphate, tributylammonium salt (PN(Me)P). N-Methylimidodiphosphoryl tetrachloride (0.53 g, 2 mmol) was treated with 1 N NaOH (19.0 ml, 19.0 mmol) at 0°C for 1 h. The reaction was then warmed to room temperature with stirring until all of the white solid had dissolved. This sample was purified by anion-exchange chromatography at 2-4°C on QAE Sephadex A-25 (HCO_3^-) using a 3-liter linear gradient of

0.1-0.7 M triethylammonium bicarbonate, pH 8.5, as eluent. Fractions were assayed for acid-labile phosphate according to the procedure of Ames (1966). PN(Me)P is eluted from the column by 0.36 M triethylammonium bicarbonate under these conditions. The appropriate fractions were pooled, 1 ml of tributylamine was added, and the resulting solution was concentrated to a syrup on a rotary evaporator using a vacuum pump and a dry ice/ethanol trap with a bath temperature below 25°C. An additional 1 ml of tributylamine was added, and the syrup was stripped of residual water and triethylammonium bicarbonate by repeated evaporation of 25-ml aliquots of absolute methanol under reduced pressure. The amount of $PN(CH_3)P$ was determined by the Ames method (1966) (1.6 mmol, 80% yield). ^{31}P NMR (20% D_2O, pH 12.6, Proton decoupled): δ 5.83 (s); (proton coupled) (quartet, J_{PH} = 9.4 Hz).

b. Preparation of Tributylammonium salt of Adenylyl diphenylphosphate

AMP . H_2O (2 mmol, free acid) was dissolved in several milliliters of absolute methanol containing 2 mmol of trioctylamine (0.0706 g) with stirring under reflux. The methanol was removed under reduced pressure with a bath temperature of 25-30°C and the residue dried by repeated addition and evaporation of three 10-ml portions of dry dimethylformamide. The residue was again dissolved in dimethylformamide (10 ml); 0.6 ml of diphenyl chlorophosphate (3.0 mmol) and 0.94 ml of tributylamine (4.0 mmol) were added, and the solution was allowed to stand at room temperature for 2 h while being protected from moisture. Solvents were removed under reduced pressure and dry ether (100 ml) was added to the residue with swirling. The flask was cooled in an ice bath for 20 min and the ether decanted. The remaining ether was removed under reduced pressure and the product stored in a stoppered flask at -20°C until used.

c. Preparation of the Triethylammonium salt of AMPPN(Me)P

The tributylammonium salt of PN(Me)P (2.4 mmol) was dissolved in 10 ml of DMF and 30 ml of pyridine. Tributylammonium adenylyl diphenylphosphate (2.0 mmol), dissolved in 10 ml of DMF, was added to this solution dropwise over a 15-min period with stirring. The reaction mixture was allowed to stand at room temperature for 30 min with stirring and then solvents were removed under reduced pressure as before. The residue was extracted with cold ether (70 ml), and the ether was decanted after standing in an ice bath for 20 min. The remaining ether was removed under pressure, and the residue was dissolved in 100 ml of cold water containing 0.8 ml of concentrated ammonium hydroxide. The cloudy solution was extracted with 100 ml of ether, and the clear water layer was applied to a 2.5 x 70 cm column of DEAE Sephadex (A-25, HCO_3^-) at 4°C with a 2400 ml linear gradient eluent of 0 to 0.6 M TEAB. The fractions were detected by their uv absorbance at 275 nm, and AMPPN(Me)P was localized by PEI-cellulose TLC. The fractions with the same R_f value as ATP were pooled and evaporated to dryness under reduced pressure at a bath temperature below 20°C. The dry residue was purified again using HPLC (ODS column). The fraction containing AMP·PN(Me)P was dried as before, and the crude product, dissolved in small amounts of cold water, was again passed through a 1 x 50-cm column of DEAE Sephadex A-25 with a 1200 ml linear gradient of 0 to 0.6 M TEAB as eluent. The procedure was the same as in the primary separation, and the appropriate fractions were collected and lyophilized to dryness. The triethylammonium salt of AMPPN(Me)P, a white fluffy powder, was obtained (140 mg, 0.15 mmol, 7.6% yield). ^{31}P NMR (D_2O, ^1H broadband decoupling on, pH 11.58) δ 2.85 (d, Jpp = 23 Hz, γ-P), -6.46 (app. t, J = 23 Hz, β-P), -11.04 (d, J_{PP} = 23 Hz, α-P). High resolution LSIMS (M-1, free acid): calcd for $C_{11}H_{18}N_6O_{12}P_3$: 519.0196; found: 519.0176.

d. Preparation of the Triethylammonium Salt of AMPN(Me)P

The tris(tributylammonium) salt of PN(Me)P (522 mg, 0.7 mmol) was dissolved in 1.0 ml of CH_3CN, and 211 mg (0.5 mmol) of 5'-tosyladenosine was added. The mixture was allowed to react for 36 h under N_2 at room temperature, and then the CH_3CN was removed under vacuum. The residue was dissolved in 40 ml of cold H_2O containing 0.3 ml concd aq. NH_3. This solution was extracted with 3 x 40 ml diethyl ether. The clear aqueous layer was loaded onto a DEAE Sephadex A-25 column (HCO_3^-, 3 x 30 cm) and eluted with a 2.5-liter linear gradient of 0-0.6 M triethylammonium bicarbonate buffer (pH 8.5). The separation was followed by analysis using PEI-cellulose TLC, the fractions with the same R_f value as ADP were pooled, and the resulting solution was lyophilized to dryness. The triethylammonium salt of AMPN(Me)P (59 mg, 0.08 mmol, 16% yield) was obtained. It gave a single spot with R_f value = 0.5 on PEI-cellulose TLC using 0.5 M triethylammonium bicarbonate buffer as eluent. ^{31}P NMR (D_2O, pD = 11.6, ^1H broad band decoupling off): δ 6.95 (s (broad), α-P); 3.38 (q, J = 8.5 Hz, β-P); (^1H broadband decoupling on): δ 6.95 (d, J = 18 Hz, α-P); 3.4 (d, J = 18 Hz, β-P). High resolution LSIMS (free acid mass, M-1): calculated for $C_{11}H_{17}N_6O_9P_2$: 439.0513; found: 439.0548.

e. Preparation of the Triethylammonium Salt of Adenosine 5'-[α,β–N-Methylimido]triphosphate (AMPN(Me)PP)

The triethylammonium salt of AMPN(Me)P (37 mg, 0.05 mmol) and phosphocreatine (400 mg, 1.6 mmol) were dissolved in 15 ml of buffer solution (0.2 M glycine, 12 mM $Mg(OAc)_2$, pH 9), and then creatine kinase (6 mg) in 0.5 ml of Hepes buffer (pH 7.6) was added. The solution was kept at room temperature for 36 h and then purified as described above using chromatography on the DEAE Sephadex A-25 column. Fractions were monitored using PEI-cellulose TLC, and those with the same R_f value as ATP were pooled and lyophilized to dryness to give 18 mg of the triethylammonium salt of

AMPN(Me)PP (40% yield). It gave a single spot with an R_f value = 0.2 on PEI-cellulose TLC using 0.5 M triethylammonium bicarbonate buffer as eluent. ^{31}P NMR (D_2O, pD = 12.6, ^1H broad band decoupling off): δ 4.5 (m, broad, α-P); -5.8 (d, J = 21 Hz, γ-P); -7.3 (m, broad, β-P). (^1H broad-band decoupling on): δ 4.6(d, J = 21 Hz, α-P); -5.9 (d, J = 22 Hz, γ-P); -7.3(app. t, J = 21 Hz, β-P). High resolution LSIMS (free acid mass, M-1): calcd: 519.0196; found: 519.0187.

f. Preparation of Adenosine 5'-[α,β-Imido]diphosphate (AMPNP)

The anhydrous Adenosine (1 mmol) were stirred in dry triethyl phosphate (5 ml) at -15°C, and 1 equivalent of dichlorophosphoryl iminotrichlorophosphorane (1 mmol) in 0.5 mL of dry triethyl phosphate was added dropwise. The mixture was stirred at -15°C for 1 hour and then 25 ml of 0.1 N NaOH was quickly added to the mixture under vigorous stirring at 0°C. The mixture was extracted with 3 aliquots of 25 mL ethyl acetate, and the water layer was evaporated to about 10 ml. The remaining water solution was applied to a DEAE Sephadex A-25 column (HCO_3^- form, 3 x 30 cm) and eluted with a linear TEAB buffer (pH 8.5) of 0 to 0.6 M at 4°C. The appropriate fractions were collected and evaporated to dryness. AMPNP was converted into their sodium salts using sodium iodide in acetone. The yield was 42% of the theoretical. It gave a single spot (R_f = 0.5) on PEI-TLC with 0.5 M TEA-HCO_3^- eluent. ^{31}P NMR (D_2O, pD = 12, ^1H broad band decoupling on): δ 2.76 (s, α-P); -1.05 (s, β-P). High resolution LSIMS (free acid mass, M-1): calculated for $C_{10}H_{15}N_6O_9P_2$: 425.0371; found: 425.0351.

g. Preparation of Adenosine 5'-[α,β-imido]triphosphate (AMPNPP)

AMPNP (0.06 mmol) was incubated for 24 h at 5°C with phosphocreatine (100 mg), creatine kinase (5 mg) in glycine buffer (pH 9), and 12 mM $Mg(OAc)_2$. AMPNPP

was then purified as described in the preparation of AMPN(Me)PP, and a yield of 77% was obtained. It gave a single spot (R_f = 0.2) on PEI-TLC with 0.5 M TEA-HCO_3^- eluent. ^{31}P NMR (D_2O, pD = 12, ^1H broad band decoupling on): δ 0.55 (1 P, d, J = 6 Hz, α-P); -5.66 (1 P, d, J = 21 Hz, γ-P); -11.07 (1 P, dd, J = 6, 21 Hz, β-P) . High resolution LSIMS (free acid mass, M-1): calculated for $C_{10}H_{16}N_6O_{12}P_3$: 505.0038; found: 505.0024.

IV. Results and Discussion

A. Synthesis and Characterization of AMPPN(Me)P

AMPPN(Me)P was originally prepared by Dr. Mark A. Reynolds (Ph. D. Dissertation, 1984). It proved to be very difficult to purify , and the final purification required three chromatographic steps. The pH dependence of its ^{31}P NMR spectrum is discussed below.

B. Synthesis and Characterization of AMPN(Me)P and AMPN(Me)PP

AMPN(Me)P was prepared by using the general methodology of Davisson *et al.* (1987), starting with commercially available 5'-tosyladenosine. A $J_{^{31}P-^{31}P}$ value of 8.5 Hz was found. Interestingly, no $J_{^{31}P-^{31}P}$ was observable for the corresponding nonmethylated AMPNP. AMPN(Me)P was found to be a respectable substrate for rabbit muscle creatine kinase (Table 2-1), and this permitted its conversion to AMPN(Me)PP. As with AMPPN(Me)P, its ^{31}P NMR spectrum showed large $J_{^{31}P-^{31}P}$ coupling constants (22 Hz), similar to those of ATP.

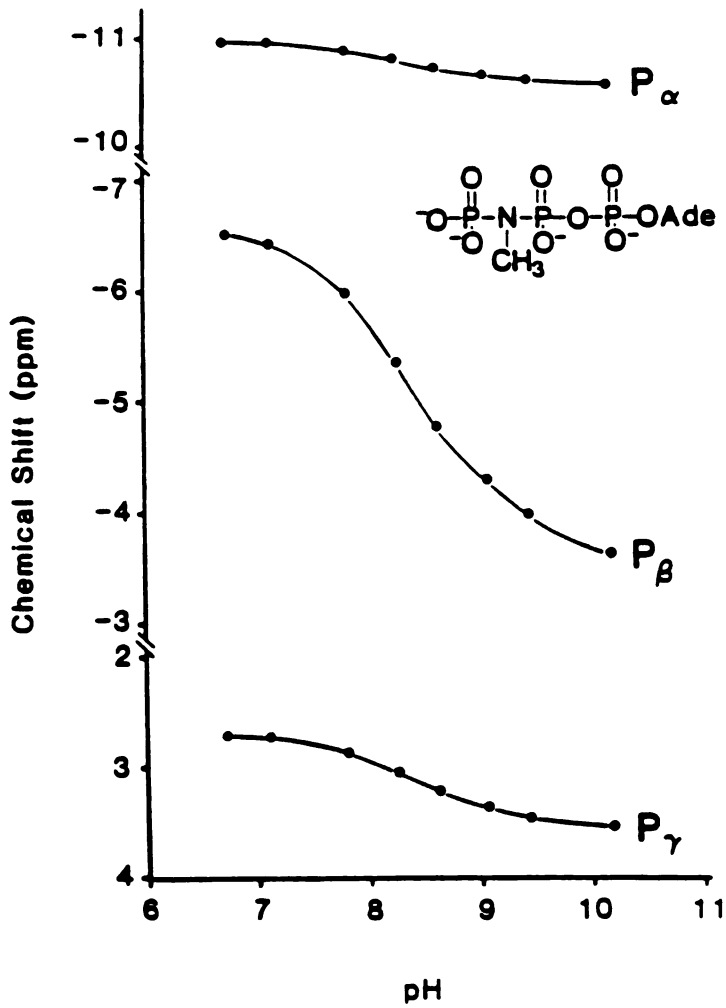


Fig. 2-2. ^{31}P NMR titration curves for the α–, β–, and γ–phosphoryl groups of AMPPN(Me)P [From Dr. Mark A. Reynolds's Dissertation, (1984)]

C. pH-Titration Curves Using ^{31}P NMR Spectroscopy

The pH-titration curves for the α-, β-, and γ-phosphorus resonances of AMPPN(Me)P are of particular interest. Previous studies reported similar pH-titration curves for AMPPNP (Reynolds et al., 1983; Tran-Dinh et al., 1975; Jaffe et al., 1978). For AMPPNP, the β-phosphorus shows by far the greatest shift of the three upon titration in the physiological pH range, even though it was clearly established by pH titrations using ^{17}O NMR that only the oxygens of the γ-phosphorus are protonated in the pH range 5-12 (Reynolds et al., 1983).

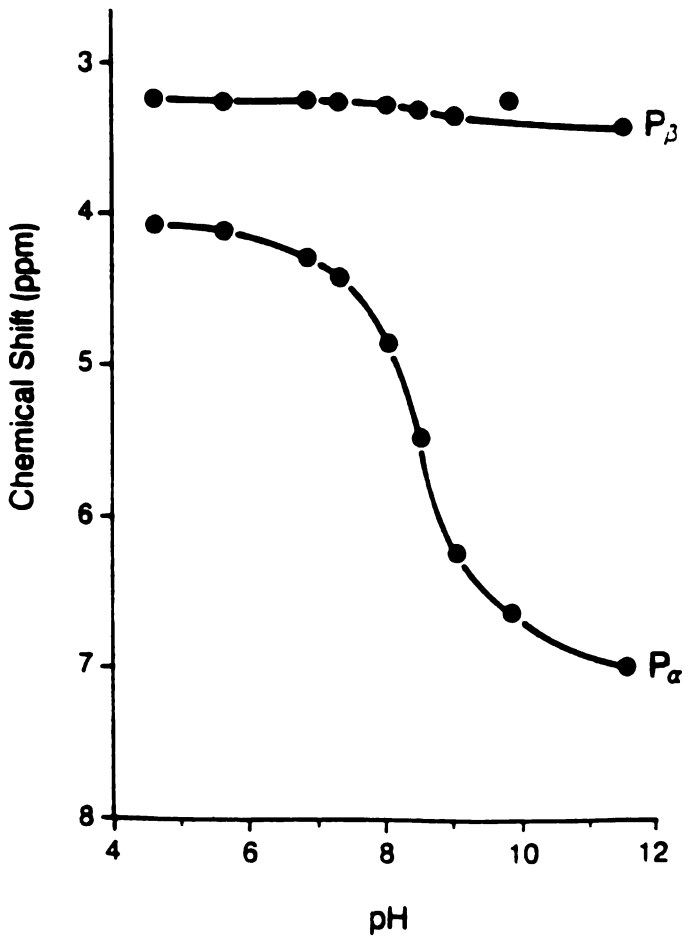


Fig. 2-3. ^{31}P NMR titration curves for the α– and β–phosphoryl groups of AMP(NMe)P

Thus the observed ^{31}P NMR shift of the β-phosphorus is an indirect effect and evidently has nothing to do with direct protonation of the oxygens on the β-phosphorus. In Fig. 2-2 are shown the corresponding pH-titration curves for AMPPN(Me)P. It closely resembles that for AMPPNP. Thus, whatever is causing the β-phosphorus to shift so dramatically is operating in both compounds. Also, since AMPPN(Me)P can be considered as a "locked" imido tautomer of AMPPNP (Reynolds et al., 1983), this similar pH-titration behavior lends further support to the earlier conclusion that AMPPNP exists in solution largely, if not exclusively, as the imido tautomer shown in Fig. 1-2.

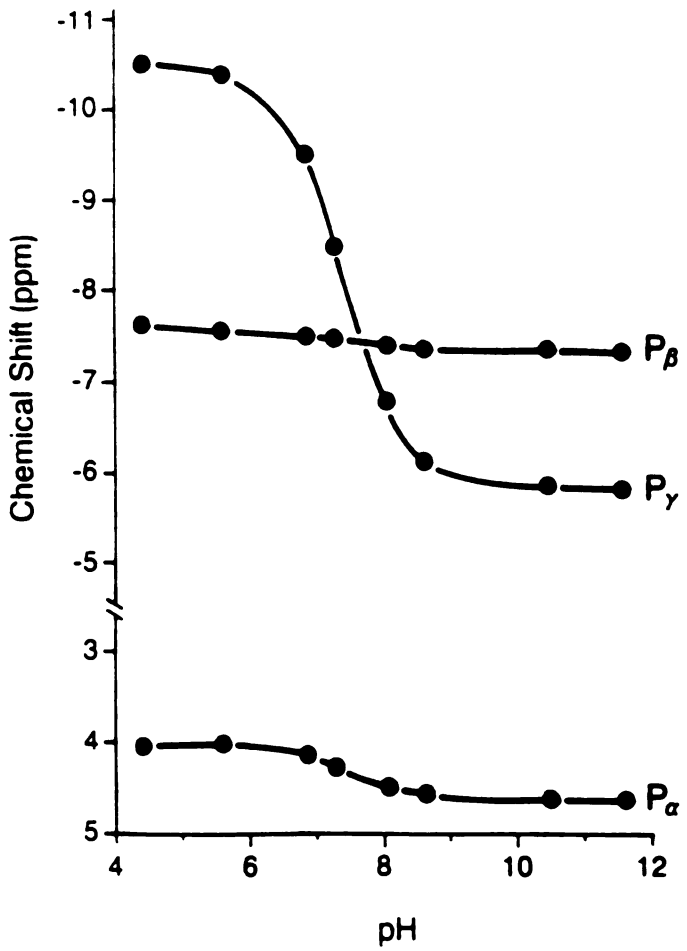


Fig. 2-4. ^{31}P NMR titration curves for the α–, β–, and γ–phosphoryl groups of AMPN(Me)PP

pH-titration curves for both AMPN(Me)P and AMPN(Me)PP, again measured using ^{31}P NMR, are shown in Figs. 2-3 and 2-4, respectively. They provide convincing evidence for the proposed structures.

D. Enzyme Kinetic Studies in the Creatine Kinase Reaction

In Table 2-1 are shown V_{max}, K_m, and V_{max}/K_m values for some of these purine nucleotide analogs. AMPPN(Me)P appears to be rejected by the enzyme since we could detect activity neither as a substrate nor as an inhibitor. Both AMPN(Me)P and AMPN(Me)PP were substrates, however. AMPN(Me)P showed ca. 40% of the V_{max} value of ADP under the same conditions, whereas its K_m value was substantially higher. Accurate values for these kinetic parameters could be detected using the coupled assay with hexokinase for which the AMPN(Me)PP produced is also a substrate. In the reverse direction, however, correspondingly accurate kinetic parameters could not be measured since the AMPN(Me)P generated was not a sufficiently good substrate for pyruvate kinase. Nevertheless, AMPN(Me)PP was shown to generate phosphocreatine from creatine using the PEI-cellulose TLC assay system of Rowley and Kenyon (1974).

The kinetic data for AMPNP as a substrate in the creatine kinase reaction are also included in Table 2-1. Comparisons of these data with those for the corresponding N-methylated analog indicates, not too surprisingly, that creatine kinase is susceptible to steric hindrance from the N-methyl group. On the other hand, the reasonably high relative V_{max} value for AMPN(Me)P shows its potential for interesting interactions with other ADP-utilizing enzymes in future studies.

Table 2-1

V_{max} and K_m values for ATP, ADP, and their corresponding imido and N-methylimido analogs in the creatine kinase reaction

Compounds	V_{max} (mM . min^{-1})	K_m (mM)	V_{max}/K_m(min^{-1})
	Forward direction (ATP consumption)		
ATP	0.790 ± 0.056	0.094 ± 0.009	8.40 ± 1.00
AMPNPP	N.D.[a]	N.D.[a]	
AMPN(Me)PP	N.D.[a]	N.D.[a]	
	Reverse direction (ATP production)		
ADP	0.297 ± 0.024	0.012 ± 0.002	24.8 ± 4.58
AMPNP	0.248 ± 0.017	0.386 ± 0.029	1.902 ± 0.081
AMPN(Me)P	0.129 ± 0.019	1.71 ± 0.35	0.075 ± 0.009

[a] *Not detectable in the coupled enzyme assays used. With the PEI-cellulose TLC assay (Rowley et al., 1974), however, phosphocreatine was unequivocally detected as a product when the analogs replaced ATP as substrates.*

3

Specificity of S-Adenosylmethionine Synthetase for ATP Analogues Mono- and Disubstituted in Bridging Positions of the Polyphosphate Chain

I. Abstract

The entire family of ATP analogues that are either mono- or disubstituted with imido and methylene bridges in the polyphosphate chain of ATP have been investigated as potential substrates and inhibitors of S-adenosylmethionine synthetase (ATP:L-methionine S-adenosyltransferase). The disubstituted analogues adenosine 5'-(α,β:β,γ-diimidotriphosphate) (AMPNPNP) and adenosine 5'-(α,β:α,β'-diimidotriphosphate) ($AMP(NP)_2$) have been synthesized for the first time, and a new route to adenosine 5'-(α,β:β,γ-dimethylenetriphosphate) (AMPCPCP) has been developed. S-Adenosylmethionine synthetase catalyzes a two-step reaction: the intact polyphosphate chain is displaced from ATP, yielding AdoMet and tripolyphosphate, followed normally, but not obligatorily, by hydrolysis of the tripolyphosphate to pyrophosphate and orthophosphate. Uniformly, the imido mono- or disubstituted derivatives are both better substrates and better inhibitors than their methylene counterparts. AMPNPNP reacts rapidly to give a single equivalent of product per active site, but subsequent turnovers are at least 1000-fold slower, enabling it to be used to quantify enzyme active site concentrations. In contrast, AMPCPCP is not detectably a substrate ($<10^{-5}$ of ATP). $AMP(NP)_2$, a branched isomer of linear AMPNPNP, was not a substrate but was a linear

competitive inhibitor, >100 fold more potent than ADP, indicating a reasonable degree of bulk tolerance at the α-phosphoryl group binding site. Adenosine 5'-(α,β-imidotriphosphate) (AMPNPP) is a surprisingly potent inhibitor as well as substrate, with an inhibition constant that is ~60-fold less than the K_m for ATP, and is an ~1000-fold better inhibitor than adenosine 5'-(α,β-methylenetriphosphate) (AMPCPP). These findings reinforce the notion that imido analogues of ATP are more suitable analogues of ATP than their corresponding methylene compounds.

II. Introduction

The only known biosynthetic route to S-adenosylmethionine arises from a reaction catalyzed by S-Adenosylmethionine (AdoMet) synthetase (ATP:L-methionine S-adenosyltransferase) catalyzes a two-step reaction in which ATP and methionine first combine to form AdoMet and tripolyphosphate; the tripolyphosphate is then hydrolyzed to PP_i and P_i (which originates as the γ-phosphoryl group of ATP) before product release (Mudd, 1973; Tabor & Tabor, 1984). In the presence of the ATP analogue AMPPNP multiple turnovers of AdoMet formation can occur without polyphosphate bond cleavage (Markham et al., 1980); therefore, AdoMet synthetase is a rare enzyme for which the influence of substitution in both the α,β- and the β,γ-bridging positions of the polyphosphate chain of ATP on enzyme affinity and activity can be assessed. Furthermore, there is the possibility that an ATP analogue containing nonhydrolyzable bonds in both the α,β- and β,γ-bridges would allow direct measurement of the equilibrium constant for the formation of AdoMet and tripolyphosphate (analogue) from ATP (analogue) and methionine, a chemical reaction for which the thermodynamics have not been characterized. In reactions of monosubstituted nonhydrolyzable ATP analogues such as AMPPNP, the tripolyphosphate derivative formed can reorient so that the P-O-P linkage occupies the β,γ-phosphoryl group site; thus, the tripolyphosphate analogue can be

hydrolyzed to P_i and the corresponding pyrophosphate derivative, preventing determination of the equilibrium constant for the first step of the overall AdoMet synthetase reaction. An ATP analogue substituted in both the α,β,- and β,γ-bridge positions could obviate this problem.

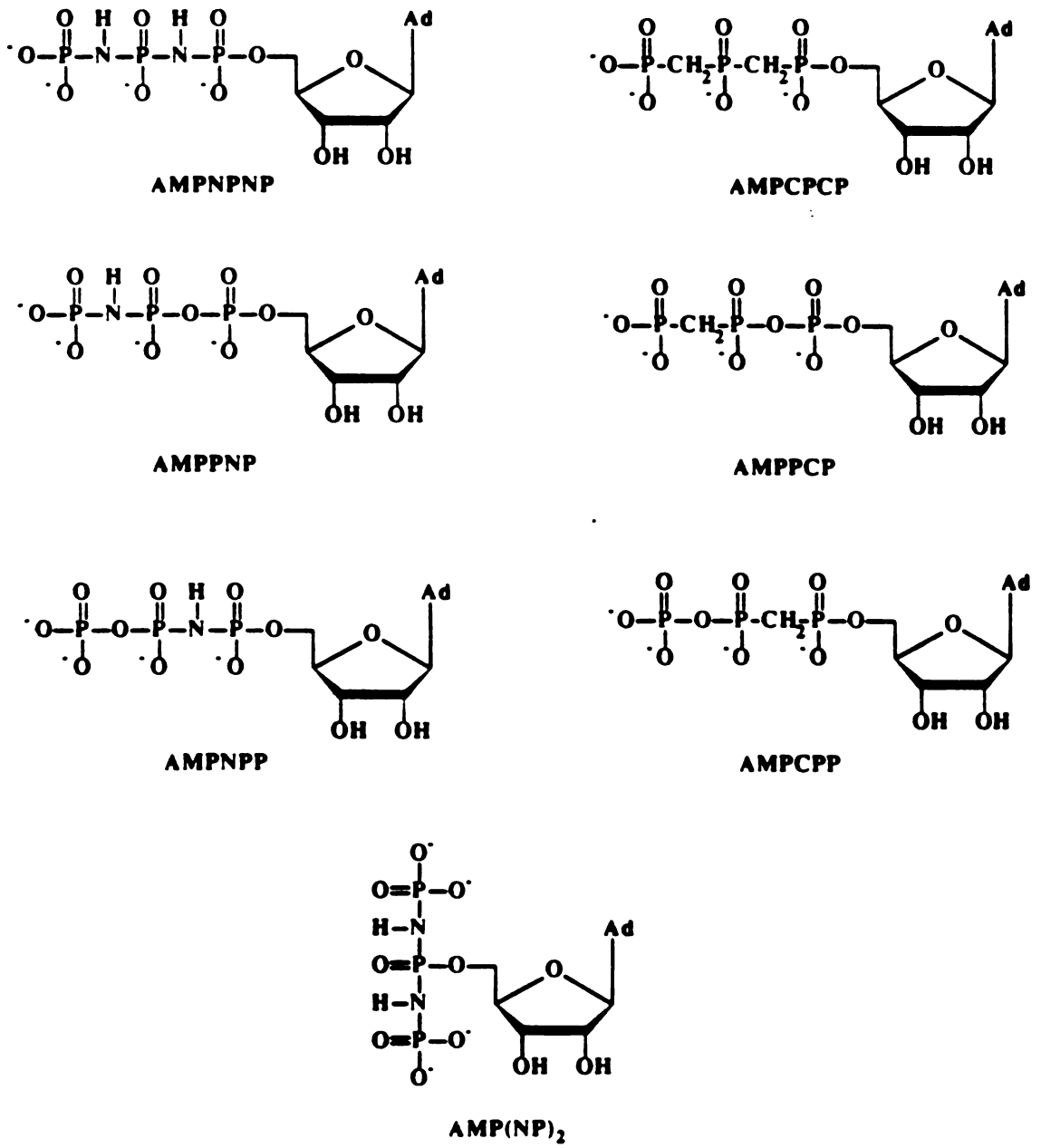


Fig. 3-1. Structures of the imido and methylene analogues of ATP

Recent synthetic advances have been developed that permit ready access to a broad variety of ATP analogues that are nonhydrolyzable at both the α,β- and β,γ-positions (Davisson et al., 1987; Ma et al., 1988, 1989). In this chapter we compare existing analogues as substrates and inhibitors of AdoMet synthetase, present for the first time a synthetic method for preparation of the bis-imido derivatives AMPNPNP and $AMP(NP)_2$, and provide a new route to the bis-methylene analogue adenosine 5'-(α,β:β,γ-dimethylenetriphosphate) (AMPCPCP). The structures of all of these ATP analogues are shown in Figure 3-1. Behaviors of the mono and disubstituted imido and methylene analogues as substrates and inhibitors of AdoMet synthetase are compared.

III. Experimental

A. Materials and Methods

^{31}P NMR spectra were obtained at 79.5 MHz on a Nicolet NTCFT-1180 NMR spectrometer, a sweep width of 4000 Hz, a probe temperature of 25°C, and ^{1}H broad-band decoupling were used in ^{31}P NMR measurements. Chemical shifts were determined relative to 85% H_3PO_4 with positive shifts being downfield from the reference. High- and low- resolution liquid secondary ion mass spectra were taken on a Kratos MS-50 mass spectrometer and a negative ion probe was used to measure the mass of the M-1 peak.

PEI- cellulose plates were obtained from J. T. Baker Co. Other chemicals were from Aldrich. AMPCPP, AMPPCP, and AMPPNP were purchased from Sigma and showed single spots on PEI-cellulose thin-layer chromatography. [methyl-^{14}C]Methionine (51.8 mCi/mmol) was purchased from New England Nuclear.

B. Synthesis

a. Trichloro[(dichlorophosphoryl)imino]phosphorane

Trichloro[(dichlorophosphoryl)imino]phosphorane was synthesized by the method of Emsley et al. (1971) and used as a precursor for the synthesis of both AMPNPNP and AMP(NP)2.

b. [P,P-Dichloro-N-(dichlorophosphinyl)phosphinimyl]-phosphorimidic trichloride

[P,P-Dichloro-N-(dichlorophosphinyl)phosphinimyl]-phosphorimidic trichloride was prepared as described by Riesel and Somieski (1975). Trichloro-[(dichlorophosphoryl)imino]phosphorane (17.8 g, 66 mmol) was dissolved in 65 ml of dry s-tetrachloroethane cooled to 0°C in an ice bath. 1,1,1,3,3,3-hexamethyldisilazane(10.9 g, 68 mmol) dissolved in 15 ml of s-tetrachloroethane was added to the stirring solution. After the mixture had been stirred at 60°C for 12 h, phosphorus pentachloride (14.4 g, 69 mmol) was added, and the reaction was continued at 120°C for another 12 h. Solvent was removed under vacuum, and product was purified by vacuum distillation at 165°C (0.5 Torr). Colorless crystals formed after cooling to room temperature and weighed 17.5 g (69% yield). ^{31}P NMR ($CDCl_3$): d 4.18 (1 P, d, J = 32 Hz); -10.49(1 P, d, J = 30 Hz), -19.57 (1 P, t, J = 31 Hz).

c. Pentasodium Salt of Diimidotriphosphate

The pentasodium salt of diimidotriphosphate was synthesized by mixing [P,P-dichloro-N-(dichlorophosphinyl)phosphinimyl]phosphrimidic trichloride (1.86 g, 4.8 mmol) with 20 ml of 0.5 N HCl and stirring at room temperature overnight. Solvent was removed below 30°C in vacuum. The residue was separated on a QAE-Sephadex column (3

x 50 cm) eluted with a 0-0.6 M gradient of triethylammonium bicarbonate, and solvent was removed as before. Four aliquots of 50 ml of methanol were added and then evaporated to remove triethylammonium bicarbonate and remaining water. The residue was passed through a Sp-Sephadex column (Na^+ form, 2 x 50 cm). The fractions containing PNPNP were pooled, and a white powder weighing 1.25 g (71% yield) was obtained. The compound gave a single spot (R_f = 0.3) on PEI-cellulose thin-layer chromatography (Rowley & Kenyon, 1974) using 1.0 M TEA-HCO_3^- eluent. ^{31}P NMR (D_2O, pD 7.5): δ -0.12 (2 P, d, J = 5 Hz, -1.82 (1 P, t, J = 5 Hz). LSIMS spectrum showed the expected parent ion of the free acid form [255 (M-1)].

d. Adenosine 5'-(α,β:β,γ-diimidotriphosphate) (AMPNPNP) and Adenosine 5'-(α,β:α,β'-diimidotriphosphate) (AMP(NP)$_2$)

AMPNPNP and AMP(NP)$_2$ were prepared by an adaptation of the method of Davisson et al. (1987). The pentasodium salt of PNPNP (399 mg, 1 mmol) was passed though a Sp-Sephadex C-25 column (H^+ form, 2 x 50 cm) and the appropriate fractions were pooled and neutralized with tetrabutylammonium hydroxide to pH 8. Solvent was removed, and the residue was dried by repeated evaporation of three aliquots of 50 ml of methanol and two aliquots of 30 ml of dry acetonitrile. The resulting tetrabutylammonium salt of PNPNP was dissolved in dry acetonitrile, and 5'-tosyladenosine (211 mg, 0.5 mmol) was added. After the mixture had been stirred at room temperature for 26 h with protection from moisture, the solvent was removed, and the residue was dissolved in 20 ml of H_2O. The solution was loaded on a Sp-Sephadex column (H^+ form, 2.5 x 50 cm), eluted with H_2O, and 2-ml of fractions were collected. AMPNPNP were eluted first (fractions 41-45) followed by AMP(NP)2 (fractions 46-51). Both AMPNPNP and AMP(NP)2 were further purified by DEAE-Sephadex A-25 column chromatography and

converted to their respective sodium salts by the method described previously (Ma et al., 1988)

The sodium salt of AMPNPNP was obtained as a white powder and weighed 21 mg (7% yield). It gave a single spot (R_f = 0.2) on PEI-cellulose thin-layer chromatography with 0.5 M TEA-HCO_3^- eluent. ^{31}PNMR (D_2O, pD 7.8, ^{1}H broad-band decoupling on): δ 0.59 (1 P, d, J = 4.1 Hz, α-P); -0.96 (1 P, d, J = 5.1 Hz, γ-P); -4.4 (1 P, t, J = 4.5 Hz, β-P). ^{31}P NMR (D_2O, pD 7.8, ^{1}H broad-band decoupling off): δ 0.59 (1 P, m, α-P); -0.96 (1 P, t, J = 5.1 Hz, γ-P); -4.4 (1 P, t, J = 4.5 Hz, β-P). The ^{31}P NMR pH titration curves (Ma et al., 1989) were fully consistent with the structure. High resolution LSIMS (free acid mass, M - 1): calculated for $C_{10}H_{16}O_{11}N_7P_3$, 504.0917; found 504.0915.

$AMP(NP)_2$, as its sodium salt, was also isolated as a white powder and weighed 17 mg (5.7% yield). It showed a single spot (R_f = 0.2) on PEI-cellulose thin-layer chromatography with 0.5 M TEA-HCO_3^- eluent. ^{31}P NMR (D_2O, pD 4.8, ^{1}H broad-band decoupling on): δ 5.21 (1 P, t, J = 7.5 Hz, α-P); -3.99 (2 P, d, J = 7.5 Hz, β,β'-P). ^{31}P NMR (D_2O, pD 4.8, ^{1}H broad-band decoupling off): δ 5.2, (1 P, m, α-P); -3.98 (2 P, d, J = 7.5 Hz, β,β'-P). ^{31}P NMR pH titration curves were fully consistent with the proposed structure. The liquid secondary ion mass spectrum gave a molecular weight of 505, as expected.

e. Adenosine 5'-(α,β-imidotriphosphate) (AMPNPP)

AMPNPP was synthesized enzymatically from AMPNP and phosphocreatine in the same way as described previously (Ma et al. 1988), but AMPNP was prepared by the alternative method of Tomasz et al. (1988). ATP contamination was ruled out by examining AMPNPP in the RNA polymerase reaction (Promega Biotec, 1988).

f. Adenosine 5'-(α,β:β,γ-dimethylenetriphosphate) (AMPCPCP)

AMPCPCP was synthesized in a similar fashion to AMPNPNP. The pentasodium salt of PCPCP was prepared by the method described by Trowbridge et al. (1972). The sodium salt (364 mg, 0.5 mmol) was then converted into its tetrabutylammonium salt and treated with 5'-tosyladenosine (211 mg, 0.5 mmol). The AMPCPCP was purified in the same way as described in the AMPNPNP synthesis. The product, as a sodium salt, was a white powder and weighed 54 mg (18% yield). The liquid secondary ion mass spectrum of the free acid form of AMPCPCP showed an expected parent ion (M - 1, 502). ^{31}P NMR (D_2O, pD 7.7, ^{1}H broad band decoupling on): δ 27.8 (1 P, t, J = 9.5 Hz, β-P), 18.5 (1 P, d, J = 10 Hz, α-P) 15.3 (1 P, d, J = 8.8 Hz, γ-P). The ^{31}P NMR titration curves were consistent with the structure.

C. Enzyme Assay

AdoMet synthetase was purified to homogeneity from *Escherichia coli* strain DM22pK8 as described previously (Markham et al., 1980; Markham, 1981). Enzyme concentrations are expressed as active site concentrations using a subunit molecular mass of 41.9 kDa.

AdoMet synthetase assays measured the incorporation of [methyl-^{14}C]methionine into AdoMet (Markham et al., 1980). At the high (up to 0.5 mM) enzyme levels used in this study, 5% of single turnover can readily be measured by this assay. Experiments were performed at 22°C.

IV. Results and Discussion

A. Synthesis and characterization of ATP analogues.

Although PNPNP has been well characterized (Quimby et al., 1960; Irani & Callis, 1961; Nielson & Pustinger, 1964; Pollard et al, 1964; Töpelmann et al., 1979), AMPNPNP has not been previously reported. It was synthesized by treating 5'-tosyladenosine with the tetrabutylammonium salt of PNPNP as described for similar syntheses by Davisson et al. (1987). In the course of purification two products were obtained, the expected AMPNPNP and a second nucleoside imidotriphosphate derivative. Further characterization of the second product showed it to be $AMP(NP)_2$, a compound in which the adenosine moiety is attached to the central phosphorus of the triphosphate chain. In contrast, reaction of 5'-tosyladenosine with tripolyphosphate according to the method of Davisson et al. (1987) yield only ATP with no detectable isomeric $AMP(OP)_2$ (<1%). A search of the literature revealed that the ATP isomer $AMP(OP)_2$ has only been described once and it was shown to be a substrate for neither hexokinase nor *E. coli* aminoacyl-tRNA synthetase, the only enzymes with which it was tested (Kozarich et al., 1973).

In view of the recent success of the method of Davisson et al. (1987) in preparation of a variety of ATP analogues, we decided to synthesize AMPCPCP by that method since it is more convenient than the earlier method (Trowbridge et al., 1972) Properties of the compound prepared by the two methods were identical. In the synthesis of AMPCPCP from 5'-tosyladenosine and PCPCP, none of the isomeric $AMP(CP)_2$ (< 1%) could be detected by ^{31}P NMR spectroscopy.

B. Studies with AdoMet Synthetase

a. Methylene Analogues of ATP

ATP analogues containing single methylene substitutions in both the α,β- and β,γ-bridge positions of the polyphosphate chain have been known for some time (Myers et al.,

1963, 1965), and these monosubstituted derivatives are available commercially. These analogues have been used with several enzymes with a mixed record of binding productively either as substrates or as potent inhibitors (Ashman & Keech, 1975; Burger & Lowenstein, 1970; Cook and Murdoch, 1973; Cuee et al., 1968; Horak & Zalik, 1976; Krug et al., 1973; Larsen et al., 1969; Mannherz & Goody, 1976; Milner-White & Rycroft, 1983; Morley & Stadtman, 1970; Rose et al., 1979; Taketa et al., 1971) The disubstituted compound AMPCPCP, which was previously reported (Trowbridge et al., 1972), has not previously been tested with enzymes.

The results of the use of the methylene bridging analogues with AdoMet

Table 3-1: Interaction of Methylene Analogues of ATP with S-Adenosylmethionine Synthetase[a]

compound	steady-state rate (min^{-1})	K_i (mM)
ATP	96	
AMPCPP	0.02	2.0
AMPPCP	<0.003	8.5
AMPCPCP	$<5 \times 10^{-5}$	>20

a *AMPCPP and AMPPCP were tested as substrates at 10 mM. A single turnover with AMPPCP has a >4-h half time. There is <5% of a single turnover from 2 mM AMPCPCP is 2 h. Inhibition by AMPCPCP was not detectable (<5%) at 3 mM nucleotide. Inhibition experiments were performed in 50 nM Hepes/KOH, 50 mM KCl, 25 mM MgCl2, 0.1 mM ATP, and 0.5 mM methionine (K_m values for ATP and methionine are 0.12 and 0.08 mM, respectively) (Markham et al., 1980). Substrate activity tests were performed in 50 mM Hepes/KOH, pH 8.0, 10 mM MgCl, 50 mM KCl, 0.5 mM[methyl-* ^{14}C*]methionine, and nucleotide. Enzyme active site concentrations between 0.03 and 0.5 mM were used.*

synthetase are reported in Table 3-1. Both AMPCPP and AMPPCP are poor substrates for the enzyme (maximally 0.02% of the activity of ATP), and AMPCPCP has no detectable substrate activity ($<10^{-5}$ of the activity of ATP). For AMPCPP the rate of product formation is linear with time from within 10% of a single turnover, indicating that product release is not rate limiting. Although AMPPCP is clearly a substrate, the rate of even a single turnover with AMPPCP (half-time = 4 h) is so slow to prevent determination of the steady-state rate.

The monosubstituted compounds are at best poor inhibitors with K_i values at least 20 times greater than the K_m for ATP (0.12 mM under these conditions as illustrated in Fig. 3-2). At best, only extremely weak inhibition was observed with the disubstituted

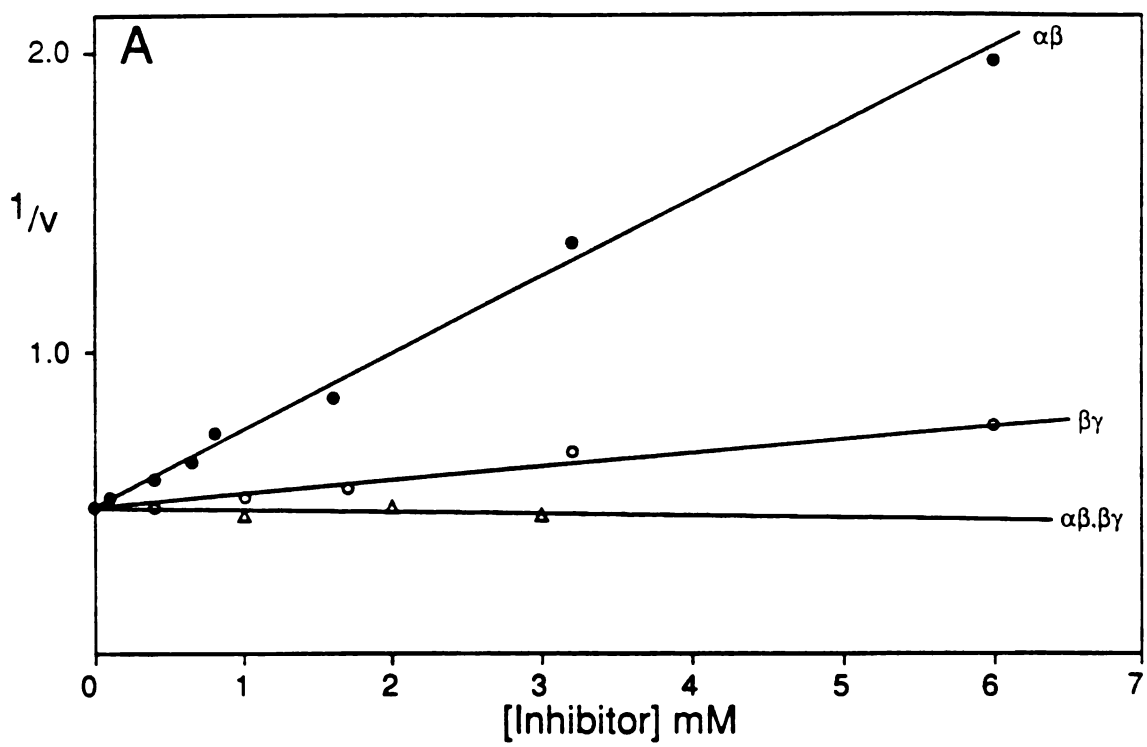


Fig. 3-2. Inhibition of AdoMet synthetase by methylene analogues of ATP

Assay solution contained 50 mM Hepes/KOH (pH 8.0), 50 mM KCl, 25 mM MgCl2, 0.1 mM ATP and 0.5 mM methionine. V is expressed as nanomoles of AdoMet formed per minute.

AMPCPCP. Between the monosubstituted compounds, AMPCPP was both the better substrate and the better inhibitor, indicating that the enzyme has a greater tolerance of geometrical distortions at the α,β-bridge position, perhaps not surprisingly since no enzymatic reactions occur at this position. The effects of methylene substitution appear to be cumulative since AMPCPCP was a poorer inhibitor (at least 2 fold) than either of the monosubstituted compounds.

b. Imido analogues of ATP

The interaction of AdoMet synthetase with the imido analogues proved to be considerably more intriguing than with the methylene analogues. At saturating concentrations (see below) all of the linear NH bridge containing ATP analogues (AMPNPP, AMPPNP, and AMPNPNP) were reasonably good substrates on a single turnover basis (Table 3-2), although AMPNPNP gave only 1% of the activity of ATP. As illustrated in Fig. 3-3 for AMPNPNP, the reactions with the above-three imido derivatives showed a burst of product formation followed by a slower steady-state rate; the burst corresponds to formation of enzyme-bound products, and the slow steady-state rate evidently results from the slow rate of product release from the [enzyme-AdoMet-triphosphate analogue] complex as was previously shown for AMPPNP (Markham et al., 1980). The burst rate for formation of enzyme-bound products were approximately the same for AMPNPP and AMPPNP; however, the burst rate was ca. 5-fold slower with the disubstituted AMPNPNP. Furthermore, while multiple turnovers were observed with AMPNPP and AMPPNP, subsequent turnovers with AMPNPNP occurred at an unmeasurably slow rate (<0.05 turnover/h). In contrast to the linear analogues, the

bifurcated $AMP(NP)_2$ had no detectable substrate activity (<10% of a single turnover in 2 h).

The inhibition of AdoMet synthetase by the four imido-ATP analogues are compared in Fig. 3-4. All were good inhibitors, although $AMP(NP)_2$ was the poorest. Further experiments with $AMP(NP)_2$ confirmed that it was a linear competitive inhibitor with respect to ATP with a K_i value of 0.22 mM compared to the ATP K_m of 0.12 mM (not shown). Remarkably, AMPNPP was a very good inhibitor of the enzyme with a K_i

Table 2-2. Interaction of Imido Analogues of ATP with S-Adenosylmethionine Synthetase

compound	burst rate (min^{-1})	steady state rate (min^{-1})	Ki (mM)
ATP	ND	96	
AMPNPP	2.8	0.10	0.0022
AMPPNP	3.5	0.04	0.09
AMPNPNP	0.6	<0.001	0.16
$AMP(NP)_2$		<0.001	0.22

ND, not determined. All compounds were tested as substrates at 1.1 mM. Other conditions are as described in Table I.

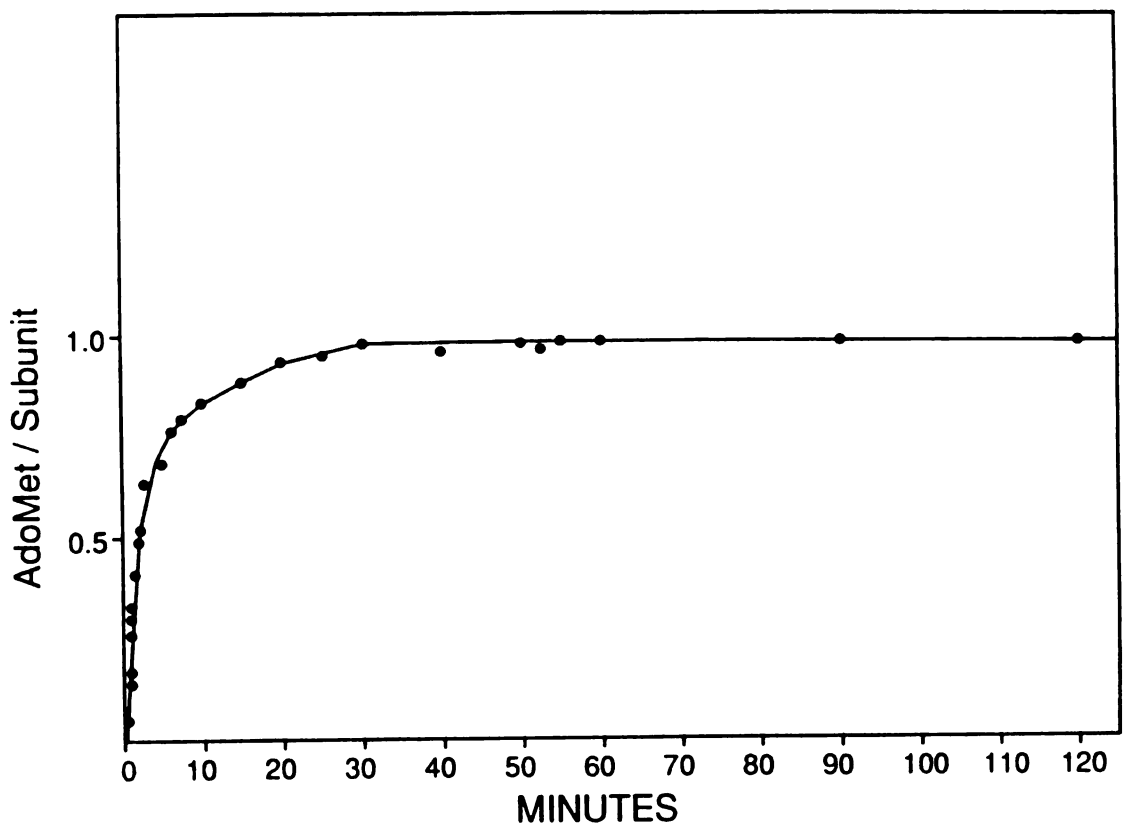


Fig. 3-3. Time Course of Product Formation from AMPNPNP and Methionine

Solutions contained 1.1 mM AMPNPNP and 0.5 mM [methyl-^{14}C]methionine in 50 mM Hepes/KOH, pH 8.0, 50 mM KCl, and 10 mM $MgCl_2$. Enzyme active site concentrations between 0.03 and 0.5 mM were used. Data are expressed as moles of AdoMet formed per mole of enzyme active sites.

value that is ~60 -fold lower than the K_m for ATP and 1000-fold less than the K_i value for AMPCPP. AMPNPP is thus the most potent known monosubstrate inhibitor of AdoMet synthetase, although certain bisubstrate analogues are better inhibitors (Kappler et al., 1988). Determination of the steady-state kinetic behavior with AMPNPP as substrate was complicated by the low K_m and low V_{max}, which necessitated use of enzyme

concentrations comparable to the substrate concentration; an upper limit of the K_m of 5 μM was obtained, consistent with the measured K_i value. In contrast to the methylene analogues, the imido substitution does not cause cumulative effects on the affinity for the enzyme since AMPNPNP is a poorer inhibitor than either AMPNPP or AMPPNP.

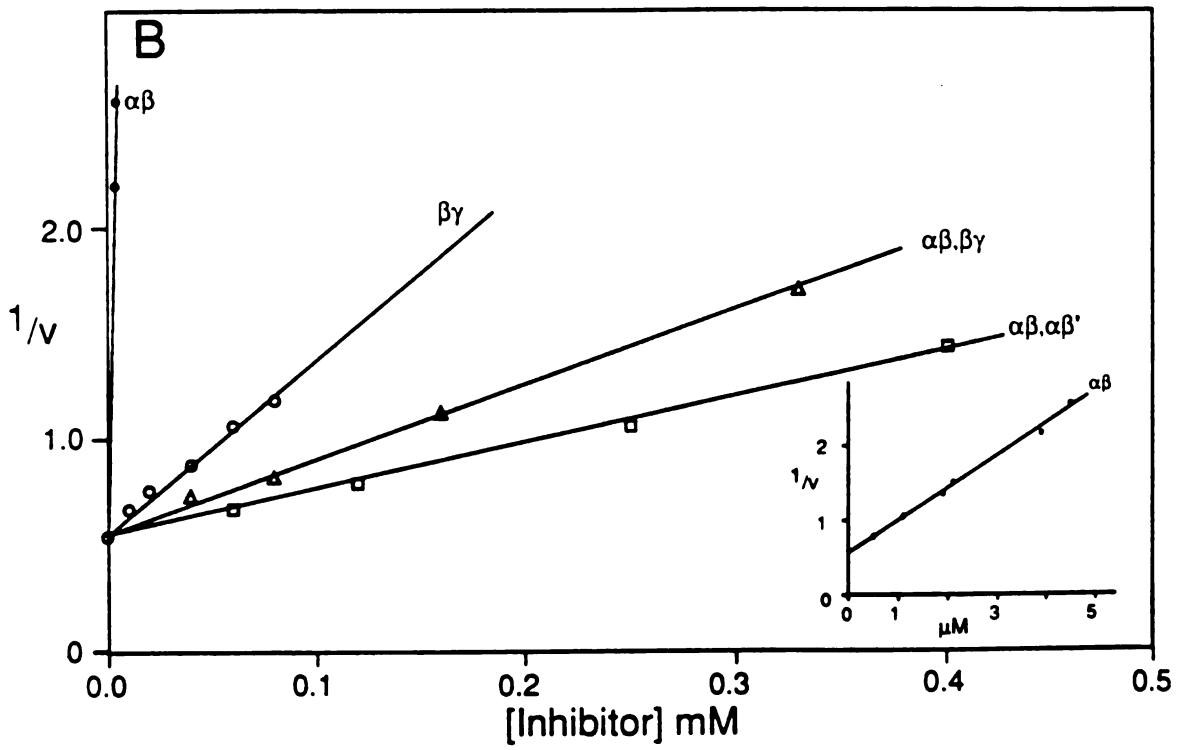


Fig. 3-4. Inhibition of AdoMet Synthetase by Imido Analogues of ATP

The insert expands the region showing inhibition by AMPNPP. V is expressed as nanomoles of AdoMet formed per minute. Other conditions are as described in Fig. 3-2.

V. Conclusions

The reaction catalyzed by AdoMet synthetase with AMPNPNP reveals several new features regarding enzyme function. The rapid formation of a single equivalent per subunit of enzyme-bound products provides evidence that the equilibrium constant for the reaction

[enzyme•AMPNPNP•methionine] = [enzyme•AdoMet•PNPNP]

lies far towards products; with AMPPNP, the equilibrium constant for the corresponding reaction was estimated to be $>10^4$ (Markham et al., 1980), and a similar estimate has been obtained for the reaction with ATP (unpublished results). Thus, at least at the active site, formation of the sulfonium compound AdoMet is a thermodynamically favorable process. The extremely slow steady-state rate of product formation from AMPNPNP and the lack of substrate activity of AMPCPCP has, however, thwarted our attempts to determine the equilibrium constant for interconversion of free substrates and products. Determination of the thermodynamics of the first step of the overall AdoMet synthetase reaction will seemingly require an yet unknown variant of AdoMet synthetase which either has lower affinity for the ternary product complex or lacks hydrolytic activity entirely; attempts to create such a variant by in vitro mutagenesis might solve this problem.

The extraordinarily slow rate of turnover with AMPNPNP provides a novel use of the compound as an active site titrant for AdoMet synthetase, in the same fashion that p-nitrophenyl esters have been used to quantify active site concentrations of serine proteases (Bender et al., 1962). Combined with the resistance of P-N bonds to most phosphatases and nucleotidases, this stoichiometric reaction with AMPNPNP should find practical use in quantifying AdoMet synthetase levels in cellular extracts.

The entire imido class of ATP analogues has substantially higher affinity for, and substrate activity with, AdoMet synthetase than the methylene class. This is presumably due to the facts that the electronegativity, lone pair availability, and geometry of the imido compounds are more similar to the natural oxygen-bridged species (Larsen et al., 1969). Since the true substrate for AdoMet synthetase is a Mg^{++}-nucleotide complex, the enhanced affinity for AMPNPP over ATP may reflect an altered distribution among the numerous metal -chelate complexes present in the solution. More insight into the behavior of these analogues may emerge from crystal structures of the metal-nucleotide complexes.

AdoMet synthetase is quite tolerant in binding analogues with substitutions along the polyphosphate chain, which is emphasized by the finding that $AMP(NP)_2$ is a competitive inhibitor with a K_i value equal to the K_m for ATP. In contrast, under the same conditions we find that 15 mM ADP causes no inhibition, indicating that ADP has a K_i value at least 200-fold higher than the K_m for ATP; since $AMP(NP)_2$ can be viewed as an α-substituted ADP analogue, this high affinity was completely unexpected. The enzyme is more conservative, however, in accepting modified compounds as substrates, and the lack of substrate activity of $AMP(NP)_2$ is not surprising since even the conservative sulfur substitution present in the two diastereoisomers of ATPαS causes complete loss of substrate activity (Markham et al., 1980) As the crystallographic studies of the enzyme progress, the reason for this remarkable affinity for $AMP(NP)_2$ may become more evident.

4

Stabilization and Activation of Recombinant Human Immunodeficiency Virus-1 Reverse Transcriptase

I. Abstract

Human immunodeficiency virus-1 reverse transcriptase-p66 is surprisingly unstable at 4°C in a typical reverse transcriptase buffer that provides complete stability when the enzyme is frozen at 70°C. The presence of an $(rA)_n(dT)_{12\text{-}18}$ template-primer in the buffer vastly improves solution stability of dilute enzyme. A reproducible ~260% activation of the enzyme is found in 1.0 M ammonium phosphate. In addition, even enzyme that had been inactivated to 13% of its initial activity could be reactivated to the same ~260% higher activity level, indicating a reversible interconversion of two forms of the enzyme. The effects of chaotropic and antichaotropic salts coupled with a prior observation of p66 monomer-dimer equilibrium provide suggestive evidence that these two forms of enzyme are monomeric and dimeric p66.

II. Introduction

Reverse transcriptase (RT) activity of human immunodeficiency virus HIV-1 is found to reside in two polypeptide chains of molecular masses 66 kDa and 52 kDa, respectively. These proteins have a common amino terminus (diMarzo Veronese et al., 1986; Lightfoote et al., 1986). The short one evidently arises from the larger one by proteolytic cleavage probably mediated by the viral protease. Dimeric association products

have been observed for pure p66 and for mixtures of p66 and p51 proteins where a 1:1 heterodimer complex consists of full length protein and its proteolytic cleavage product (Müller et al., 1989). Reverse transcriptase activity has been observed for preparations of p66, p51 and p66-p51 heterodimers (Müller et al., 1989; Lori et al., 1988). As yet there is no definitive quantitative study on activity differences between monomeric p66 and p51 and their dimeric associated forms. Müller et al. (1989) observed p66 monomers to be less active than p66 homodimers but did not quantitate the differences of activity.

We found HIV-1 reverse transcriptase-p66 to be surprisingly unstable when stored in solution in a typical reverse transcriptase buffer (RT buffer) that provides complete stability when enzyme is stored frozen at -70°C. For example, the enzyme lost 31% of its activity after only 20 hours at 4°C.

In this chapter we describe the use of both substrates and salts to stabilize HIV-1 reverse transcriptase-p66. We report greatly enhanced stabilization and activation of the enzyme upon storage in solution and provide evidence consistent with a reversible monomer-dimer equilibrium as responsible for the observed changes of enzyme activity. Practically, we have developed conditions to store p66 enzyme for prolonged periods at 4°C, thus simplifying future enzyme studies.

III. Experimental

A. Materials and Methods:

Poly(rA)$_n$ oligo(dT)$_{12-18}$ was from Pharmacia; [methyl, 1',2'-^{3}H]thymidine triphosphate was from Amersham; thymidine triphosphate, tris, glycerol, triton X-100, 2-mercaptoethanol, and disodium EDTA were from Sigma; potassium chloride was from Mallinckrodt; ammonium phosphate (dibasic) and other salts tested were from Aldrich.

Stability test salt solutions were adjusted to pH 7.5 before incubation with HIV-1 reverse transcriptase.

Reverse transcriptase buffer (RT buffer): 50 mM Tris-HCl, 50 mM potassium chloride, 20% glycerol, 0.1% Triton X-100, 2 mM 2-mercaptoethanol and 4 mM EDTA, pH 7.5.

B. Plasmid Construction

The parent yeast (*Saccharomyces cereviciae*) expression plasmid pBS24 and the yeast alcohol dehydrogenase-2/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP) hybrid promoter have been described previously (Sabin et al., 1989). For construction of the RT 5 gene encoding amino acids Pro156 to Leu715 of the HIV-1 *pol* open reading frame, we modified the previously described RT4 gene (Barr et al., 1987) using synthetic oligonucleotides. The 78mer duplex included the restriction enzyme *Asp*718 target site at amino acid Trp690 and a *Sal* 1 site immediately following the termination codon. Oligonucleotides were synthesized by the phosphoramidite method using Applied Biosystems 380A DNA synthesizers.

C. Protein Preparation

HIV-1 RT5 was prepared by a modification of the previously described procedure (Barr et al., 1987). Briefly, yeast cells transformed with plasmid pBS24RT5 were grown in leucine deficient minimal media and then transferred to yeast extract, peptone and 2% dextrose (YEPD) and grown for 48 hours. The induced yeast cells were harvested by centrifugation and lysed in 50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA, 0.1% Triton X-100. The buffer also contained the following proteinase inhibitors: PMSF (1 mM) and pepstatin, aprotinin, leupeptin and chymostatin at 1 μg/ml each. The supernatant was

fractionated by ammonium sulfate precipitation (0-50%) and the pellet chromatographed on Whatman P-11 phosphocellulose, followed by further separation on Pharmacia Mono S. Final purification was by gel filtration on Pharmacia S200 HR in 50 mM Hepes-Cl, pH 7.0, containing 10% glycerol, 1 mM EDTA, 1 mM PMSF, 0.1% n-octyl-β-D-glucopyranoside and 1 mM 2-mercaptoethanol. Protein was concentrated and the buffer was changed to RT buffer using an Amicon Centricon-30. Purified protein was stored frozen at -70°C.

D. Activation and Inactivation by Salts:

a. Activation by 1.0 M ammonium phosphate

HIV-1 RT, 20 µl (1:10 dilution in RT buffer), was mixed with 20 µl of aqueous ammonium phosphate (2.0 M) and incubated at 4°C. Aliquots, 3 µl, were removed at different incubation times. RT buffer, 87 µl, was added and 10 µl assayed for activity.

b. Activation by 0.10 to 2.25 M ammonium phosphate

Thawed stock HIV-1 RT, 3 µl, in RT buffer, was mixed with 9 µl each of aqueous ammonium phosphate (3.0 M, 2.0 M, 1.33 M, 0.8 M, 0.2 M, 0.0 M) and incubated at 4°C. A 1 µl aliquot was removed from each incubation solution at the initial time and at 72 h, diluted with appropriate volumes of RT buffer and ammonium phosphate (100 mM concentrate) to yield a total volume of 150 µl that was 33.3 mM in phosphate. Enzyme activity was then assayed using 10 µl portions.

c. Activation and inactivation by other salts

HIV-1 RT, 5 µl, (1:10 dilution in RT buffer), was mixed with 5 µl of various salt solutions (2x concentrates) listed in table 1 and incubated at 4°C. Aliquots (3 µl), were removed at initial time and 24 h and diluted with 87 µl RT buffer. Enzyme activities were determined on 10 µl portions.

d. Heat inactivation and reactivation of enzyme

HIV-1 RT, 60 µl, (1:10 dilution in RT buffer) was incubated at 37°C for 30 min. At different time intervals, 3 µl was removed and diluted with 177 µl of RT buffer. Aliquots (10 µl), of diluted HIV-1 RT were assayed. A 20 µl portion of the 37°C inactivated HIV-1 RT was cooled to 4°C, and 2.0 M ammonium phosphate was added. The remainder of the experiment was the same as reported above for activation by 1.0 M ammonium phosphate.

E. Stabilization of Enzyme by Template-Primer

a. Stabilization by 450 µM template-primer

Template-primer, 44.4 µl (6.08 mM, 33.6 A_{260} U/ml), HIV-1 RT, 10 µl (1:10 dilution in RT buffer), and RT buffer, 545.6 µl, were mixed and incubated at 4°C. Aliquots, 10 µl, were removed at different time intervals and assayed. The control experiment without template-primer was done by mixing HIV-1 RT, 10 µl (1:10 dilution in RT buffer), and 590 µl of RT buffer.

b. Stabilization by 10 to 450 µM template-primer

HIV-1 RT, 20 µl (1:100 dilution in RT buffer), was mixed with 60 µl of various concentrations of template-primer (600 µM, 330 µM, 133 µM, 67 µM, 13.3 µM, 0.0 µM)

and incubated at 4°C. An aliquuot, 40 µl, was removed from each incubation at initial time and 72 h, diluted with appropriate volumes of RT buffer and template-primer (1.0 mM concentrate) to yield solutions that were 450 µM in template-primer in a total volume of 60 µl. Activity was determined with 10 µl aliquots.

F. Enzyme Assays

All enzyme assays were run in triplicate and an average of the results was calculated for each data point. The average enzyme activity was normalized to initial activity (100%). Aliquots, 10 µl, of stability test solution were mixed with 40 µl of RT assay solution (below) and incubated at 37°C for 30 min. The reaction was quenched by addition of 800 µl of ice-cold 10 % trichloroacetic acid-10 mM sodium pyrophosphate. Precipitated polynucleotide was collected by filtration through Whatman GF/C filters. The filters were washed with three portions each of ice-cold 10% trichloroacetic acid -10 mM sodium pyrophosphate and 100% ethanol and then air dried. The filters were counted for their tritium content. RT assay solution contained 62.5 mM Tris-HCl (pH 8.5), 7.5 mM $MgCl_2$, 100 mM KCl, 10 mM DTT, 0.063% Triton X-100, 50 mM TTP, 1.25 µCi [^{3}H]TTP, and 112.5 mM (0.622 A_{260} U/ml) template-primer. RT assay solution for the template-primer stabilization experiments was the same except without template-primer.

IV. RESULTS

High purity recombinant HIV-1 reverse transcriptase-p66 was obtained from the yeast *Saccharomyces cerevisiae* (Figure 4-1). The gene for its synthesis encoded amino acids Pro156 to Leu715 of the HIV-1 pol open frame corresponding to the proposed sequence for HIV-1 reverse transcriptase-p66 (Lightfoote et al., 1986).

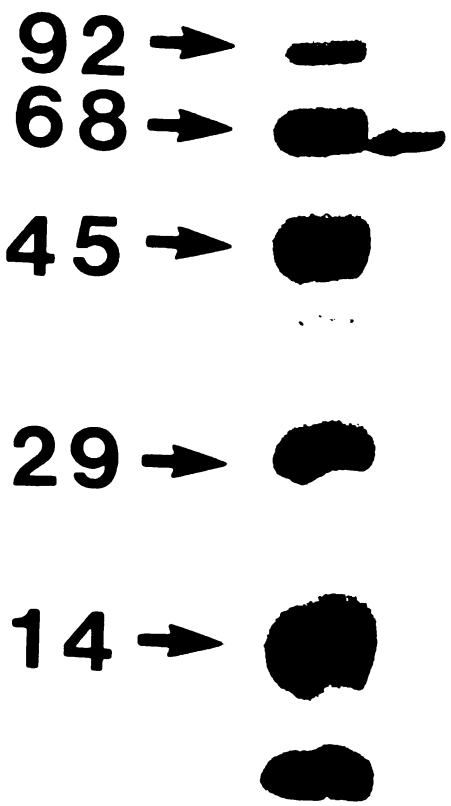


Fig. 4-1. Electrophoresis of HIV-1 reverse transcriptase-p66.
The electrophoresis was performed on 12.5% polyacrylamide with 1% SDS and stained by Coomassie blue. Left lane, molecular weight markers. Right lane, HIV-1 RT-p66.

Stability of HIV-1 reverse transcriptase-p66 in relatively dilute solutions was vastly improved by addition of hybridized 1:1 molar ratio of poly(rA) oligo$(dT)_{12\text{-}18}$ template-primer (Figure 4-2). For example, after 20 hours storage at 4°C, only 0.4% of enzyme activity was lost in the presence of 450 μM template-primer whereas 31% was lost without template-primer.

Unexpectedly, incubation of the enzyme in either 1.0 M ammonium or sodium phosphate in RT buffer, pH 7.5, caused about 260% increase of enzyme activity which

stabilized and remained relatively constant after about 72 hours at 4°C (Figure 4-2). Maximum activation of enzyme proved to be quite reproducible, yielding 257, 248, 230, 287% increases of enzyme activity in four separate experiments.

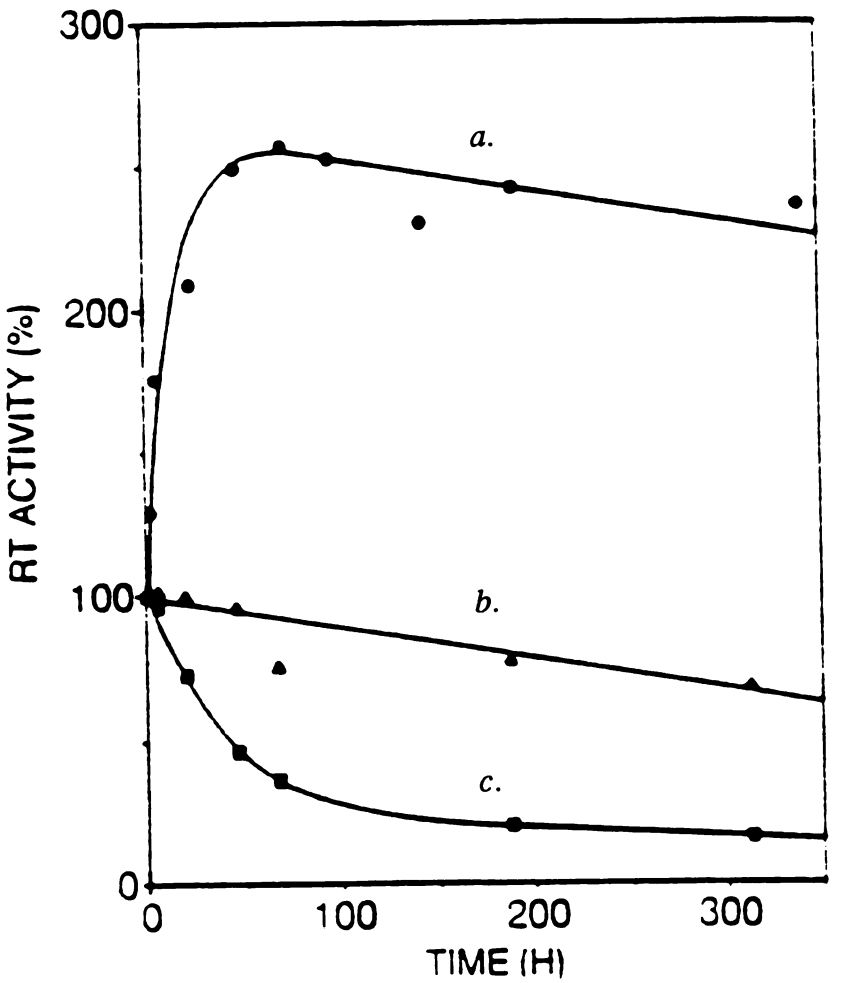


Fig. 4-2. Activation and stabilization of HIV-1 RT.

a. Activation of 1/20 dilution of stock enzyme in 1.0 M ammonium phosphate.

b. Stabilization of 1/600 dilution of stock enzyme by 450 μM template-primer.

c. Instability of 1/600 dilution of stock enzyme in RT buffer.

Concentration-dependence of template-primer stabilization and of phosphate activation is depicted in Figure 4-3. Template-primer is effective for stabilizing enzyme at

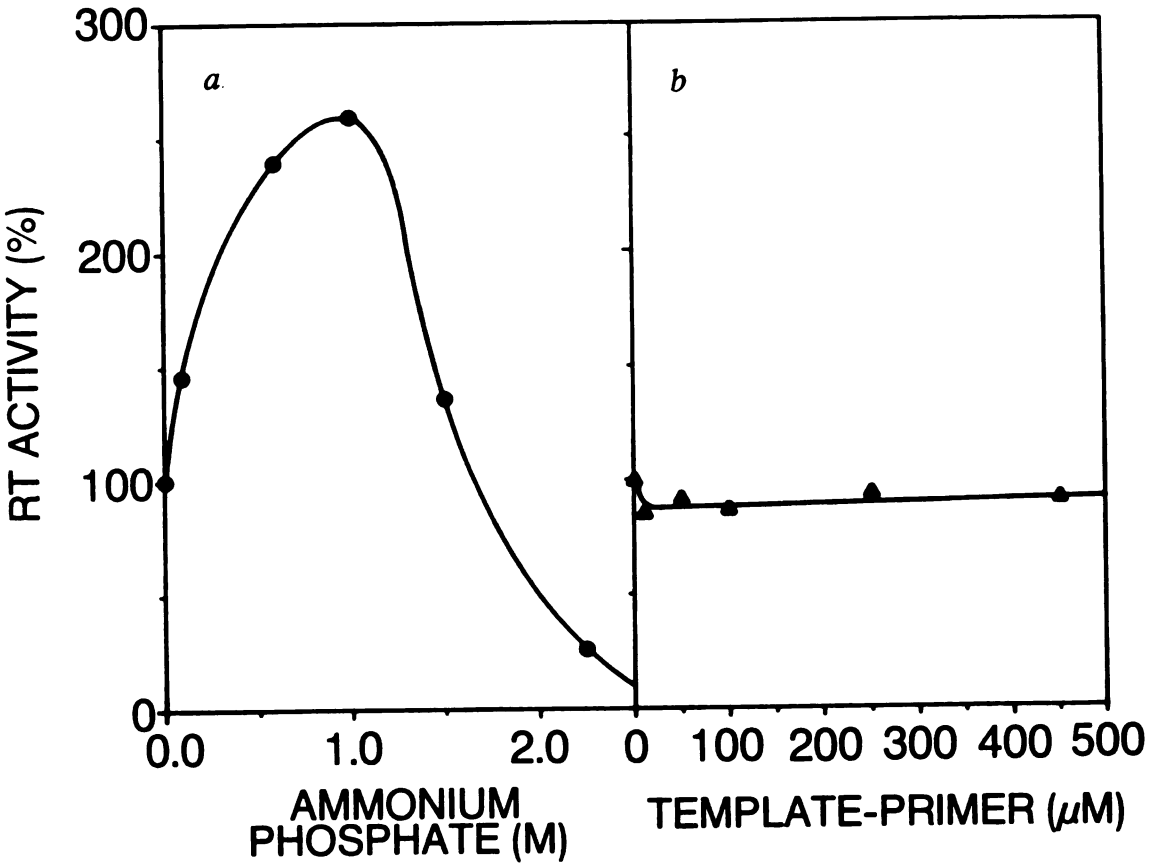


Fig. 4-3. Dependence of HIV-1 RT activation and stabilization on the concentration of phosphate or template-primer.

a. Activation of 1/20 dilution of stock enzyme in ammonium phosphate (0.10 to 2.25 M) for 72 h.

b. Stabilization of 1/20 dilution of stock enzyme in template-primer (10 to 450 μM) for 72 h.

all concentrations tested, 10 μM and above. Ammonium phosphate activates reverse transcriptase activity at all concentrations between 0.1 M and 1.5 M. Activation was most pronounced at 1.0 M. Final phosphate concentration in the enzyme assays was adjusted to 6.6 mM, a concentration that had no effect on the enzyme rate. The effect of other salts

on enzyme activity is listed in Table 4-1 in decreasing order of remaining activity after 24 hours incubation. All promoted activation similar to phosphate with the notable exception of ammonium thiocyanate which almost completely inactivated the enzyme.

Table 4-1. Effect of various salts on HIV-1 reverse transcriptase activity

Salt	Concentration[a]	Activity (%)[b]
sodium polyphosphate	15%	226
ammonium arsenate	1.0 M	220
ammonium phosphate	1.0 M	209 (252)[c]
sodium trimetaphosphate	0.37 M	207
ammonium sulfate	1.0 M	181 (257)[d]
sodium tripolyphosphate	0.27 M	164
sodium pyrophosphate	0.060 M	151
ammonium thiocyanate	0.50 M	3[e]

a. Reverse transcriptase stock solution was diluted 1/20 with salt concentrates and with RT buffer to give solutions containing the indicated salt concentrations and half concentration of RT buffer. Incubation was at 4 ℃ for 24 hours.

b. Reverse transcriptase activity was determined on 1/50 dilution of salt solutions.

c. Incubation was at 4 ℃ for 96 hours.

d. Incubation was at 4 ℃ for 144 hours.

e. Inactivation to 3% occurred in less than 4 minutes.

Enzyme that had been inactivated to 13% of its initial activity without phosphate in the buffer was restored to complete activity by subsequent addition of ammonium

phosphate to give a 1.0 M solution (Figure 4-4). In fact, reactivation proceeded beyond initial activity up to the same ~260% of its initial activity as was obtained by direct activation of stock enzyme in 1.0 M phosphate buffer.

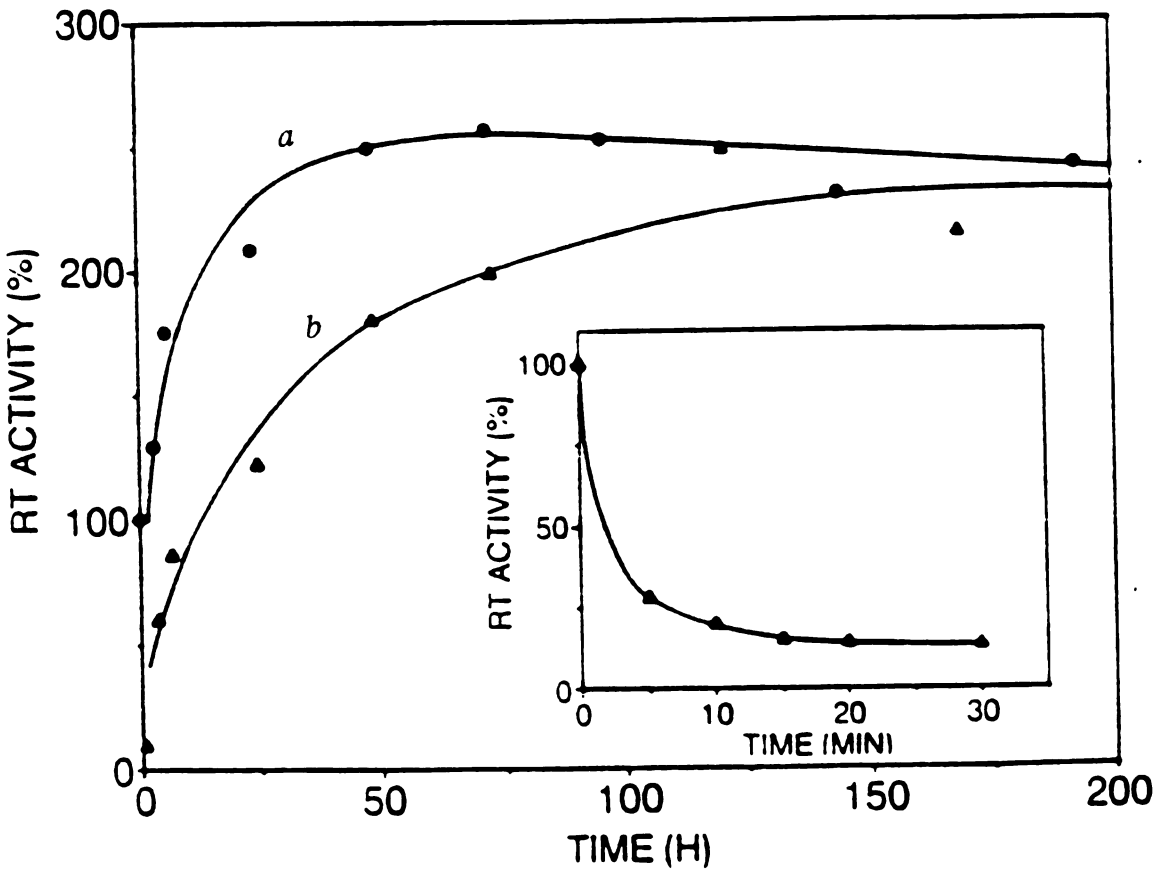


Fig. 4-4. Activation and inactivation of HIV-1 RT is reversible.

a. Activation of 1/20 dilution of stock enzyme in 1.0 M ammonium phosphate.

b. Inactivation of 1/20 dilution of stock enzyme in RT buffer at 37℃ (inset) followed by reactivation in 1.0 M ammonium phosphate at 4℃.

Lines for the ascending limbs of the activation curves are calculated for assembly of two monomeric subunits into dimer.

The rapid loss of 87% of the activity in reverse transcriptase buffer, subsequent reactivation of enzyme to an activity 2.6 times greater than the initial activity , and direct

activation of enzyme to the same 2.6 fold activity all imply that a reversible process is responsible. There appear to be two interconvertible states of the enzyme, a low activity form that has about 5% of the activity of a high activity form, and the high activity form itself. Frozen stock enzyme appears to be a mixture of these two forms.

V. Discussion

The most likely explanation for the two enzyme states is a reversible monomer-dimer equilibrium where the low activity form of the enzyme is a p66 monomer and the high activity form is a dimer. Mixtures of monomers and dimers of p66 protein have been separated by HPLC permeation chromatography where reverse transcriptase activity was found mainly in the dimer peak (Müller et al., 1989). Furthermore, a very slow equilibrium between monomers and homodimers was described when the monomer and dimer fractions were re-chromatographed.

The 5% relative enzyme activity that we can ascribe to monomers can be considered as an upper limit of monomer activity. It is conceivable that monomers are inactive. We do not know whether complete dissociation to monomers had occurred in the experiment depicted in Figure 4 or whether an equilibrium mixture was established containing a small fraction of active dimers. Our purified frozen p66 stock protein appears to be a mixture of dimers and monomers in a calculated approximate composition of 36% to 39 % dimers depending on whether monomers either have 5% of the activity of dimers or are inactive. This composition is almost identical to that previously reported (35% dimers) for prolonged storage of p66 protein at -20°C (Müller et al., 1989).

The effect of various salts upon enzyme activity provides supporting evidence for the proposed monomer-dimer equilibrium hypothesis. The greatest activation of enzyme is promoted by those anions, phosphate and sulfate, known to cause association of protein subunits (Jencks, 1969). In contrast, nearly instantaneous total loss of enzyme activity

was found upon addition of thiocyanate (Table 4-1), a chaotropic anion known to cause dissociation of subunits. The other salts listed of Table 4-1 are either polyphosphates pr phosphate analogs that would be expected to behave similarly to phosphate in promoting association of subunits.

Our observations, coupled with those of Müller et al. (1989) provide evidence to suggest that the V_{max} value reported for homodimer p66 (Lowe et al., 1988) may be lower than the true V_{max} of pure homodimer due to the probable presence of relatively inactive monomeric p66 in homodimer preparations. Homodimer of p66 is reported to have about half the activity of heterodimer p66-51 (Müller et al., 1989; Lowe et al., 1988). Our results indicate that a true comparison of the activity of p66 homodimer relative to that of p66-51 heterodimer must be done in conjunction with a determination of the state of aggregation of the two proteins.

We proposed that the high degree of stabilization noted upon addition of template-primer to enzyme (Figure 4-2) is due to its binding to the dimeric form promoting high stability of the template-primer-dimer complex and suppressing dissociation to the considerably less active monomers. Moreover, the enzyme is stable when it was incubated with template-primer in RT buffer at 37°C for 40 min. In contrast when the enzyme is stored in RT buffer at 37°C for 40 minutes, only about 12% of the reverse transcriptase activity remains. As a consequence of the high apparent stability of the template-primer-dimer complex, one can reason that template-primer ought to promote enzyme activation by assisting association to a tight complex. Lack of activation of enzyme by the template-primer (Figure 4-3) is readily explained by the vast difference in enzyme concentration between the template-primer and the phosphate stabilization experiments.

An alternate explanation of our reversible activity observations (Figure 4-4) involves reversible denaturation of p66 protein. The salt effects would be expected to be identical in such a process. Phosphate and sulfate would protect against denaturation and thiocyanate would promote denaturation (Jencks, 1969). We favor the subunit equilibrium

explanation due to the prior observation of a monomer-dimer equilibrium (Müller et al., 1989).

5

The Observed Inhibitory Potency of 3'-Azido-3'-deoxythymidine 5'-Triphosphate for HIV-1 Reverse Transcriptase Depends on the Length of the Poly(rA) Region of the Template

I. Abstract

The inhibitory potency of 3'-azido-3'-deoxythymidine 5'-triphosphate (AZTTP) against HIV-1 reverse transcriptase (HIV-1 RT) has been further evaluated. The results indicate that the previously reported low K_i value for AZTTP against HIV-1 RT (4 nM) is neither due to the direct tight binding of AZTTP to HIV-1 RT nor to the interaction of the enzyme with the intermediates or products from AZTTP incorporation, but instead is an artifact of the use of a homotemplate-primer (poly(rA):oligo(dT)). With a set of RNA's of defined sequence as templates, we demonstrate that the observed K_i value for AZTTP depends on the length of the poly(rA) region following the primer in the RNA template. The more adenosyl residues in the RNA template available for processive incorporation of TMP moieties, the lower is the observed K_i value for AZTTP. Since the potencies of new inhibitors of HIV-1 RT are usually compared to that for AZTTP, these results have important consequences for the process of the discovery of such new inhibitors that are of potential use in AIDS therapy.

II. Introduction

3'-Azido-3'-deoxythymidine (AZT), the first drug used clinically for the treatment of Human Immunodeficiency Virus (HIV) infection, is considered to be a prodrug that is converted into 3'-azido-3'-deoxythymidine 5'-triphosphate (AZTTP) by cellular kinases (Mitsuya et al., 1985; Fischl et al.,1987; Furman et al., 1986). *In vitro* kinetic studies show that AZTTP is a very potent competitive inhibitor of HIV-1 RT with an observed K_i value of 4 nM (Eriksson et al., 1989), and the therapeutic effect of AZT towards HIV infection has been attributed to this inhibition (Eriksson et al., 1989; Vrang et al., 1987; Cheng et al., 1987). The inhibitory mechanism of AZTTP is a subject of much current research, and one important question is why the observed K_i value for AZTTP is so much lower than the K_d value for TTP (about 30 μM) (Painter et al., 1991).

Since AZTTP is also a substrate for HIV-1 RT (Kedar et al., 1990; Reardon et al., 1990), any species in the reaction coordinate, such as AZTTP, its intermediates or products, might bind tightly to the enzyme/template-primer complex and cause the observed low K_i value for AZTTP. Recent advances in nucleotide synthesis have enabled us to study these factors separately, and the results indicate that the commonly observed low K_i value for AZTTP is an artifact of the use of long homopolymer of rA in the template.

III. Experimental

A. Materials and methods

^{31}P NMR spectra were obtained at 79.5 MHz on a Nicolet NTCFT-1180 NMR spectrometer, A sweep width of 4000 Hz, a probe temperature of 25 °C, and ^{1}H broad-band decoupling were used in ^{31}P NMR measurements. Chemical shifts were determined relative to 85% H_3PO_4 with positive shifts being downfield from the reference. High- and

low- resolution liquid secondary ion mass spectra were taken on a Kratos MS-50 mass spectrometer and a negative ion probe was used to measure the mass of the M-1 peak.

PEI- cellulose plates were obtained from J. T. Baker Co. Other chemicals were from Aldrich. The in vitro transcription kit was from Promega; poly(rA), $(dT)_{14}$, DNA polymerase I large fragment (Klenow), polynucleotide kinase and 4 deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP) were from Pharmacia; HIV-1 reverse transcriptase-p66 was prepared as described previously (Rowley et al., 1990).

B. Synthesis

a.Tetrasodium Imidodiphosphate

Tetrasodium imidodiphosphate was prepared as described previously (Ma et al., 1988; Ma et al., 1989; Ma et al., 1990) and was used as the precursor for the synthesis of the thymidine triphosphate analogues.

b. 5'-Imidodiphosphates of the Thymidine Analogues

5'-Imidodiphosphates of the thymidine analogues were prepared from the corresponding thymidine analogues (dT, AZT) according to the procedure we described before (Ma et al., 1990). The thymidine analogues (1 mmol) were dried, stirred in anhydrous triethyl phosphate (5 mL) at -15 °C, and 1.5 equivalent of dichlorophosphoryl iminotrichlorophosphorane (1.5 mmol) in 0.5 mL of anhydrous triethyl phosphate was added dropwise. The mixture was stirred at -15 °C for 1 hour and then 25 mL of 0.1 N NaOH was quickly added to the mixture under vigorous stirring at 0 °C. The mixture was extracted with 3 aliquots of 25 mL ethyl acetate, and the water layer was evaporated to about 10 mL. The remaining water solution was applied to a AG^{R}MP-1 anion exchange

column (Bio-Rad, 2.5 X 30 cm), which was subsequently eluted with a linear TEAB buffer (pH 8.5) of 0 to 0.5 M at 4 °C. The appropriate fractions were collected and evaporated to dryness. The 5'-diphosphates of the analogues were converted into their sodium salts using sodium iodide in acetone. The yield were 47% of theoretical for AZTMPNP and 50% for TMPNP. Both have similar UV spectra with absorption maxima at 266 nm and show a single spot on PEI-TLC with an R_f value comparable to that of TDP(0.3 M TEAB buffer as a eluent, $R_f = 0.55$). Their ^1H NMR spectra are consistent with the proposed structures. Both analogues were further characterized by LSIMS and ^{31}P NMR. For TMPNP, ^{31}P NMR (D_2O, pD = 10.3, ^1H broadband decoupling on) δ 2.18 (1 P, s, β-P), 5.61 (1 P, s, α-P). The liquid secondary ion mass spectrum (LSIMS) gave the expected molecular weight (M-1 = 400). For AZTMPNP, ^{31}P NMR (D_2O, pD = 10.5 , ^1H broadband decoupling on) δ 2.32 (1 P, s, β-P), 5.49 (1 P, s, α-P). The molecular weight from LSIMS further confirmed the proposed structure (M-1 = 425).

c. AZTMPNPP and TMPNPP

AZTMPNPP and **TMPNPP** were synthesized from their corresponding nucleoside imidodiphosphates(AZTMPNP and TMPNP). Nucleoside imidodiphosphates (Na^+ salts, 0.5 mmol) were converted into mono(tributylamonium) salts by passing through a Sp-Sephadex column (H^+ form, 2.0 x 10 cm) followed by adding one equivalent of tributylamine (0.5 mmol). The resulting solution was evaporated to dryness and dissolved in 5 mL of anhydrous DMF. The mixture was then cooled to 0 °C, and 5 equivalents of carbonyl diimidazole (2.5 mmol) in 5 mL DMF were added to the mixture with stirring under an Argon atomosphere. The reaction was allowed at 0 °C for 30 min. and at room temperature for 3 hours. Dry methanol (4 equiv.) was added, and the resulting solution was stirred for 30 min. Mono(tributylammonium) phosphate (1.5 equiv.) in 5 mL DMF was added to the mixture, and the solution was stirred for another 7 hours. The

reaction was quenched by adding 30 mL 0.1 N NaOH, and the mixture was then separated as described above for the imidodiphosphate analogues. The yield was 38% of the theoretical for AZTMPNPP and 41% for TMPNPP. Both showed similar behavior on PEI-TLC to TTP (single spot with R_f of 0.4 when 0.4 M of TEAB buffer as eluent), and the structures were elucidated by ^1H NMR, ^{31}P NMR and LSIMS . For AZTMPNPP, ^{31}P NMR (D_2O, pD = 9.9, ^1H broadband decoupling on) δ 0.93 (1 P, d, J = 7 Hz, α-P), -4.19 (1 P, d, J = 22 Hz, γ-P), -10.45 (1 P, dd, J = 7, 22 Hz, β-P).^1H NMR (D_2O) δ 7.87 (1 H, s, 6-H), 6.31 (1 H, t, J = 6 Hz, 1'-H), 4.73-4.84 (4 H, m, 3',4',5'-H), 2.5 (2 H, m, 2'-H), 1.95 (3 H, s, $-CH_3$).LSIMS gave the expected molecular weight (M-1 =505). For TMPNPP, ^{31}P NMR (D_2O, pD = 7.5, ^1H broadband decoupling on) δ 2.79 (1 P, s, α-P), -5.67 (1 P, d, J = 20 Hz, γ-P), -8.77 (1 P, d, J = 20 Hz, β-P). ^1H NMR (D_2O) δ 7.81 (1 H, s, 6-H), 6.39 (1 H, t, J = 6 Hz, 1'-H), 4.73-4.84 (4 H, m, 3',4',5'-H), 2.42 (2 H, m, 2'-H), 1.97 (3 H, s, $-CH_3$). LSIMS gave the expected molecular weight (M-1 = 480).

d. Synthesis and identification of 5'-^{32}P-3'-azido(dT)$_{15}$

$(dT)_{14}$ (Pharmacia) was 5'-labeled with ^{32}P by polynucleotide kinase according to the method recommended by the manufacturer (Pharmacia). The 5'-$^{32}P(dT)_{14}$ was then annealed with equimolar amounts of poly(rA), and the resulting template-primer was used to synthesize the needed oligonucleotide. The reaction mixture for the synthesis is the same as described in the general method for HIV-1 RT inhibition assay except for the following: 1 μM poly(rA)-5'-$^{32}P(dT)_{14}$, 30 μM AZTTP, 0.3 μg/ml HIV-1 RT(P66) and no TTP. After incubation at 37 C for 1.5 hrs., the reaction was quenched with EDTA and the needed oligonucleotide was purified by an anion exchange column (NACS PREPAC™ from Biochemical Research Laboratory) to remove unreacted AZTTP using the method recommended by the manufacturer. The oligodeoxynucleotide obtained was further

purified by preparative 20% polyacrylamide/7 M urea gel, visualized by autoradiography and the desired band was extracted by conventional methods (Ogden et al., 1987). The extracts were desalted by a Sep-Pak cartridge(Waters), characterized by electrophoresis (Fig. 5-1a) and quantitated by radioactivity measurements.

C. Enzyme Kinetics

a. Inhibition of HIV-1 RT by 5'-^{32}P-3'-azido(dT)$_{15}$:Poly(rA).

5'-^{32}P-3'-Azido(dT)$_{15}$ was mixed with poly(rA) in the annealing buffer (10 mM tris-HCl, pH 8.0, 5 mM $MgCl_2$). The mixture was heated to 90 °C for 2 min., placed at 50 °C and allowed to cool slowly over 30 min to form the terminated product, 5'-^{32}P-3'-azido(dT)$_{15}$:poly(rA). p(dT)$_{14}$ was also annealed with poly(rA) in the same manner to form the template -primer. The assay was the same as the general method for HIV-1 RT inhibition assay except that constant amounts of p(dT)$_{14}$:poly(rA) (0.5 μM) and ^{3}H TTP (20 μM) were used and variable amounts of 5'-^{32}P-3'-azido(dT)$_{15}$:poly(rA) were used.

b. General Method for HIV-1 RT Inhibition Assay

The assay mixture (20 μl) for the kinetic study contained: 50 mM Tris-HCl (pH 8.0), 6 mM MgCl, 8 mM DTT, 80 mM KCl, 0.4 μM poly(rA)-oligo(dT), 60 ng/ml of HIV-1 RT (P66) , different template-primers and inhibitors, and variable amounts of ^{32}P-TTP (4 to 20 μM, 0.2-1 μCi/20 μl). After incubation at 37 °C for 30 min., the mixtures were quenched by 5 μl of 0.5 M EDTA, and the template-primers were collected on DE-81 paper. The incorporation rate of TMP moieties was measured by counting ^{32}P on the DE-81 paper, and the K_i values were calculated from double reciprocal plots.

c. Preparation of RNA Templates with Different Length of Poly(rA) Region

The RNA templates, $r(A)_1$, $r(A)_{10}$ and $r(A)_{50}$, were prepared from 3 sets of synthetic ds DNA fragments containing the following sequences:

```
3'-TCGACCGAAT   AGCTTTAATT   ATGCTGAGTG   ATATCCCTCT  GGCCTTCGAA
5'-AGCTGGCTTA   TCGAAATTAA   TACGACTCAC   TATAGGGAGA  CCGGAAGCTT

3'-CGTACGGACG       (T)n      CCAGCTGAGA   TCTCCTAGGG G
5'-GCATGCCTGC       (A)n      GGTCGACTCT   AGAGGATCCC C
```

(n = 1, 10, 50)

The positive strands of the DNA's were synthesized by Biomolecular Resource Center at UCSF, annealed with the primer described in Fig. 5-3 (e), and converted into ds DNA by Klenow fragment using conventional methods. Each of the ds DNA fragments was used as the template for T7 RNA polymerase in vitro transcription. The synthesis and purification of the RNA's were performed using protocols provided by the manufacturer (Promega), and the concentrations of the RNA's obtained were measured by the amount of ^3H UMP incorporation. The template-primers were prepared by annealing each RNA with equimolar amounts of synthetic oligodeoxynucleotide(d_{21} mer) in the same way as described in the section on inhibition of HIV-1 RT by 5'-^{32}P-3'-azido$(dT)_{15}$:poly(rA). The resulting template-primers were ready for kinetic studies.

d. Inhibition of HIV-1 RT by AZTTP with different RNA's as templates

The assay conditions are the same as described in the general method for HIV-1 RT inhibition assay; 0.4 μM of 4 different template-primers (the template-primers in Fig. 5-3 (e) and poly(rA):$(dT)_{12\text{-}18}$) and variable amounts of AZTTP were used.

e. Inhibition of HIV-1 RT by AZTMPNPP and TMPNPP

0.4 μM of poly(rA):$(dT)_{12-18}$ was used as a template:primer and variable amounts of AZTMPNPP or TMPNPP were used as inhibitors. General methods for the HIV-1 RT inhibition assay were then employed.

IV. Mathematical Model

Based on the fact that AZTTP can be incorporated into a DNA chain almost as effectively as TTP (Reardon, et al., 1990), we assume that AZTTP has the same kinetic properties as TTP except for the fact that AZTTP can terminate the growing DNA chain. We consider one cycle of polymerization. This cycle includes the association of an enzyme molecule with a template-primer, the incorporation of the first TMP moiety by a distributive event, the subsequent incorporation of TMP moieties by processive events, and the dissociation of the enzyme from the template-primer. The number of TMP moieties (N_t) incorporated during one cycle will be

$$N_t = n \cdot L \qquad (1)$$

where n is the number of the enzyme molecules in the assay system and L is the length of the poly(rA) region of the template. In a specific case where equal amounts of AZTTP and TTP are present in the system, the number of TMP moieties (N_a) incorporated during one cycle of polymerization can be described as

$$N_a = n \cdot \left(\sum_{i=1}^{L} (0.5)^i\right) \qquad (2)$$

We define a term T_a, which is the sum of the time required for the enzyme to dissociate from and reassociate with the template-primer. Similarly, terms T_b and T_c are defined as the times required for one TMP moiety to be incorporated in distributive and processive events, respectively. The total time for one cycle of polymerization can be calculated, and

the cycle numbers (C_t for the system without AZTTP and C_a for the system with equal amounts of AZTTP) can be described as

$$C_t = 1/(n \cdot T_a + n \cdot T_b + n \cdot T_c \cdot (L-1)) \quad (3)$$

and

$$C_a = 1/(n \cdot T_a + n \cdot T_b + n \cdot T_c \cdot (\sum_{i=2}^{L} (0.5)^i) \quad (4)$$

The total numbers of TMP moieties incorporated during one unit of time (M_t for the system without AZTTP and M_a for the system with equal amounts of AZTTP) are given by equations 5 and 6.

$$M_t = N_t \cdot C_t = n \cdot L/(n \cdot T_a + n \cdot T_b + n \cdot T_c \cdot (L-1)) \quad (5)$$

and

$$M_a = N_a \cdot C_a = (n \cdot (\sum_{i=1}^{L} (0.5)^i) / (n \cdot T_a + n \cdot T_b + n \cdot T_c \cdot (\sum_{i=2}^{L} (0.5)^i) \quad (6)$$

Since the ratio of M_a to M_t reflects the % activity, the % inhibition can be described as

$$\% \text{ inhibition} = 1 - M_a/M_t$$

$$= 1 - ((T_a + T_b + T_c(L-1) / (T_a + T_b + Tc \cdot (\sum_{i=2}^{L} (0.5)^i)) \quad (7)$$

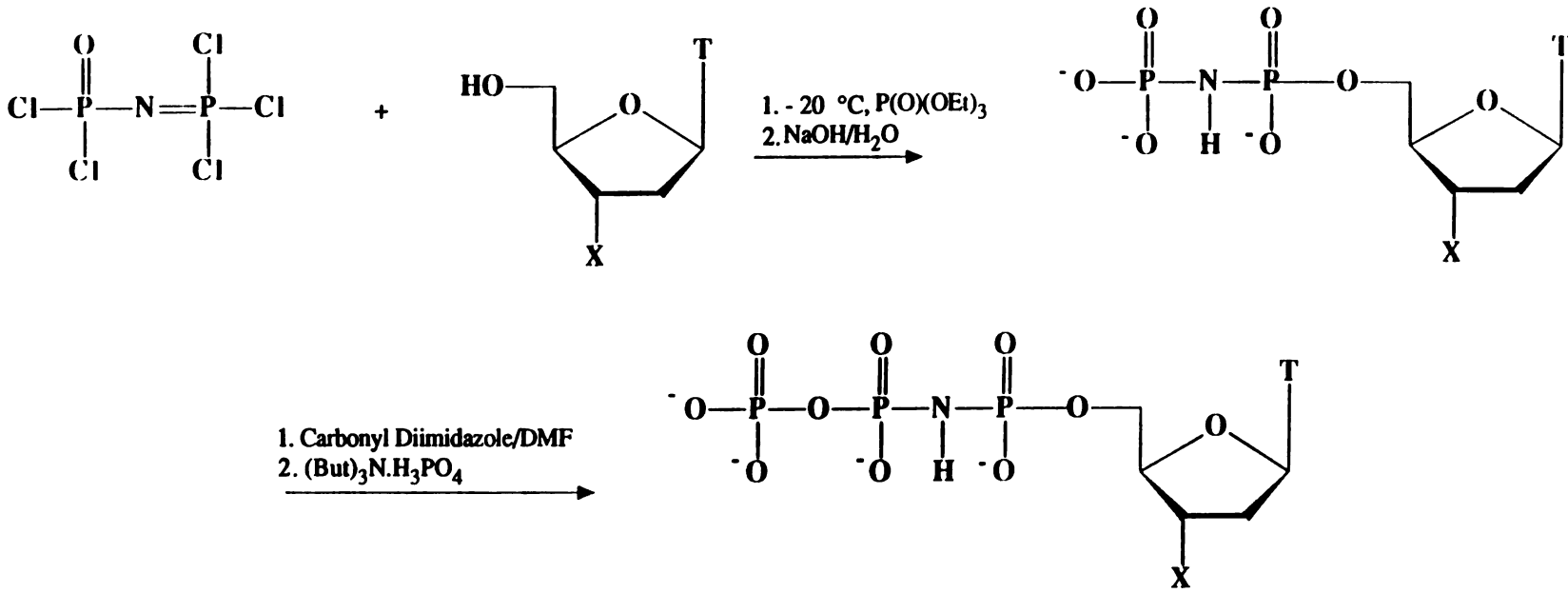
Since the time required for the dissociation of the enzyme from the template-primer is ~90-fold longer than that for TMP moiety incorporation (Reardon, et al., 1990), we define an ideal system where $T_a >> T_b$ & T_c. In this system, if L is restricted in a certain range (i. e., L is sufficiently small so that the T_c terms remain negligible in eq. (7)), the equation can be simplified to:

$$\% \text{ inhibition} = 1 - (\sum_{i=1}^{L} (0.5)^i)/L \quad (8)$$

which can be further reduced to:

$$\% \text{ inhibition} = 1 - (1 - (0.5)^L)/L \quad (9)$$

as shown in results and discussion.



X = OH TMPNPP
X = N_3 AZTMPNPP

Scheme 5-1. Synthesis of TTP analogues

V. Results and Discussion

The classical method to evaluate the affinity of an competitive inhibitor with an enzyme is to compare the K_i value for the inhibitor with either the K_m or K_d value for the substrate. Though both the K_m and K_d values have been reported, we do not think they are comparable since AZTTP is also a substrate of HIV-1 RT and the K_i value for AZTTP reflects a combination of substrate binding effects, intermediate binding effects and the effects of product release. One approach to test the relative binding of TTP and AZTTP to HIV-1 RT is to compare the K_i values for their non-hydrolyzable analogues. These analogues cannot be converted into products; hence, their K_i values should be about the same as their K_d values. The relative affinity of TTP vs. AZTTP for the enzyme can be obtained by comparison of the K_i values for these analogues. Accordingly, we have synthesized the nonhydrolyzable thymidine 5'-(α,β-imido)triphosphate analogues, namely thymidine 5'-(α,β-imido)triphosphate (TMPNPP) and 3'-azido-3'-deoxythymidine 5'-(α,β-imido)triphosphate (AZTMPNPP) (Scheme 5-1). Since the PNP bond closely resembles the POP bond in bond angle, bond length and other properties but is resistant to enzymatic cleavage (Larsen et al., 1969), it has been possible to use these analogues to study the effect of 3'-substitution on binding to HIV-1 RT. As expected, these new analogues are typical competitive inhibitors of HIV-1 RT. The kinetic studies showed that AZTMPNPP, the analogue that mimics AZTTP, binds about 10 fold more *weakly* to HIV-1 RT than does TMPNPP (Table 5-1), not more strongly as might be expected for a direct tight binding process. These rather surprising results suggest that the potent inhibition of HIV-1 RT by AZTTP is not due to the tight binding of AZTTP to the enzyme and that the K_i value for AZTTP is not directly related to its K_d value in this case.

We next examined the possibility that the low observed K_i value for AZTTP is a result of formation of a tightly associated complex of the enzyme with the termination

Table 5-1: K_i values for the Non-Hydrolyzable Analogues

Analogue	Type of inhibition	K_i value (μM)
TMPNPP	competitive inhibition	2.4 ± 0.1
AZTMPNPP	competitive inhibition	22 ± 1.3

product from AZTTP incorporation. To examine this hypothesis, a typical AZTTP termination product, 5'-^{32}P-3'-azido$(dT)_{15}$, was synthesized (Fig. 5-1a) and annealed to poly(rA). The resulting hybrid was used in an inhibition study against HIV-1 RT. As shown in Fig. 5-1b, 50% inhibition of HIV-1 RT was achieved only when the concentration of 5'-^{32}P-3'-azido$(dT)_{15}$:poly(rA) reached about the same level as that of the normal template-primer. Under the same conditions, less than 10 nM of AZTTP gave rise to 50% inhibition (data not shown). AZTTP (10 nM) could only convert 2% of the normal template-primer into template-3'-azidoprimer under our assay conditions (data not shown). This low concentration of AZTMP moiety-terminated template-primer would generate insignificant inhibition. These results suggest that the low K_i value for AZTTP is *not* due to the chain terminated products that accumulate during the time course of the assays.

Another possibility for the low K_i value for AZTTP is that the intermediates formed during AZTTP incorporation might bind to the enzyme much more tightly than the corresponding intermediates from the incorporation of AZTTP. This question can be answered by analyzing the k_{cat} values for AZTTP and TTP from the experiments performed by Reardon and Miller (1990). They simplified the kinetic model by summarizing the steps subsequent to TTP (or AZTTP) addition as k_{cat} and use a defined sequence DNA primed RNA template in the experiments. The k_{cat} values for TTP and AZTTP are 0.025 s^{-1} and 0.071 s^{-1}, respectively. Since the binding of the terminated

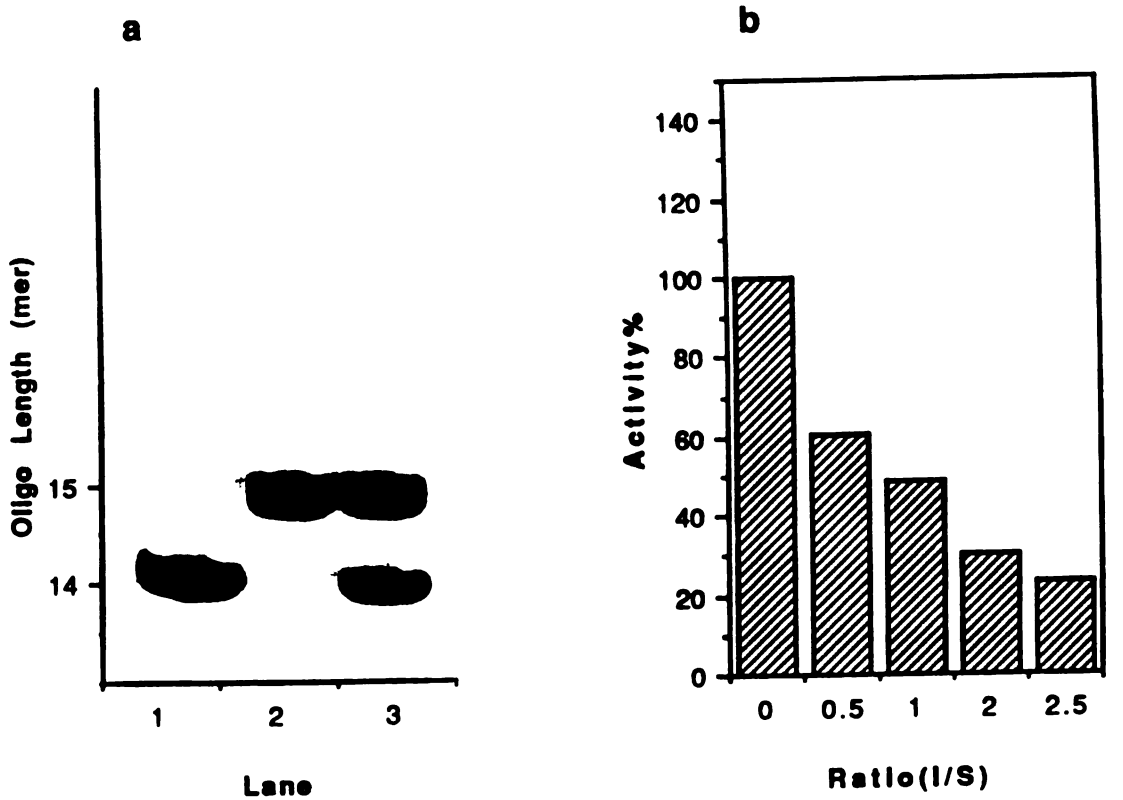


Fig. 5-1. Terminated product inhibition of HIV-1 RT

a. *Identification of 5'-^{32}P-3'-azido$(dT)_{15}$. The autoradiogram was obtained from 20% polyacrylamide/7 M urea gel. lane 1, 5'-^{32}P$(dT)_{14}$; lane 2, 5'-^{32}P-3'-azido$(dT)_{15}$; lane 3, 5'-^{32}P$(dT)_{14}$ + 5'-^{32}P-3'-azido$(dT)_{15}$.*

b. *Inhibition of HIV-1 RT by 5'-^{32}P-3'-azido$(dT)_{15}$. The I/S on the horizontal axis of the figure represents the molar ratio of 5'-^{32}P-3'-azido$(dT)_{15}$:poly(rA) to 5'-^{31}P$(dT)_{14}$:poly(rA).*

template-primer to the enzyme is about the same as that for normal template-primer as discussed above, the relative k_{cat} values for TTP and AZTTP would reflect the relative affinity of the enzyme for the intermediates from the incorporation of TTP or AZTTP. The k_{cat} values suggest that the intermediates from AZTTP incorporation do not contribute significantly to the K_i value for AZTTP.

It is worthwhile to note that the observed K_i value for AZTTP with HIV-1 RT has varied greatly in previous reports (Reardon et al., 1990). A K_i value of 160 nM was reported when a primed RNA of mixed but defined sequence was used as the template-primer (Reardon et al., 1990). Under the same conditions, but using poly(rA):oligo(dT) as the template-primer, the same authors obtained a quite different K_i value for AZTTP (35 nM) (Reardon et al., 1990). This means that the difference between the K_i value for AZTTP and the K_d value for TTP evidently depends on the nature of the template-primer.

By analyzing our results and the reported kinetic data, we hypothesize that the low K_i value for AZTTP is an artifact. All of the low K_i values for AZTTP reported in the literature were obtained with poly(rA):oligo(dT) as a template-primer (Furman et al., 1986; Kedar et al., 1990; Reardon et al., 1990; Eriksson et al., 1989; Vrang et al., 1987; Cheng et al., 1987; St. Clair et al., 1987; Ono et al., 1986; Matthes et al., 1987; Vrang et al., 1988; White et al., 1989; Starnes et al.,1988). In this assay system, ^{3}H-TTP was used as a substrate, and the inhibition was measured by the incorporation of ^{3}H-TMP moieties. Since the polymerization catalyzed by HIV-1 RT is a processive event, once an AZTMP moiety is incorporated into the growing DNA chain, ^{3}H-TMP moiety incorporation for the remaining processive events is prevented, and the enzyme has to dissociate from the template-primer. We reason that the longer the processive event in a given system, the lower the observed K_i value for AZTTP; the longer the homotemplate, the lower the observed K_i value for AZTTP. This effect of template length and sequence on the inhibition potential can be described as

$$\% \text{ inhibition} = 1 - (1 - (0.5)^L)/L$$

where L is the length of the poly(rA) region of a template. As we can see from the equation and the plot for this model (Fig. 5-2), the percent inhibition increases from 50% to 90% as the length of the poly(rA) region increases from 1 to 10. Thus, the K_i value for a chain terminator should decrease as the length of the poly(rA) region of the template increases.

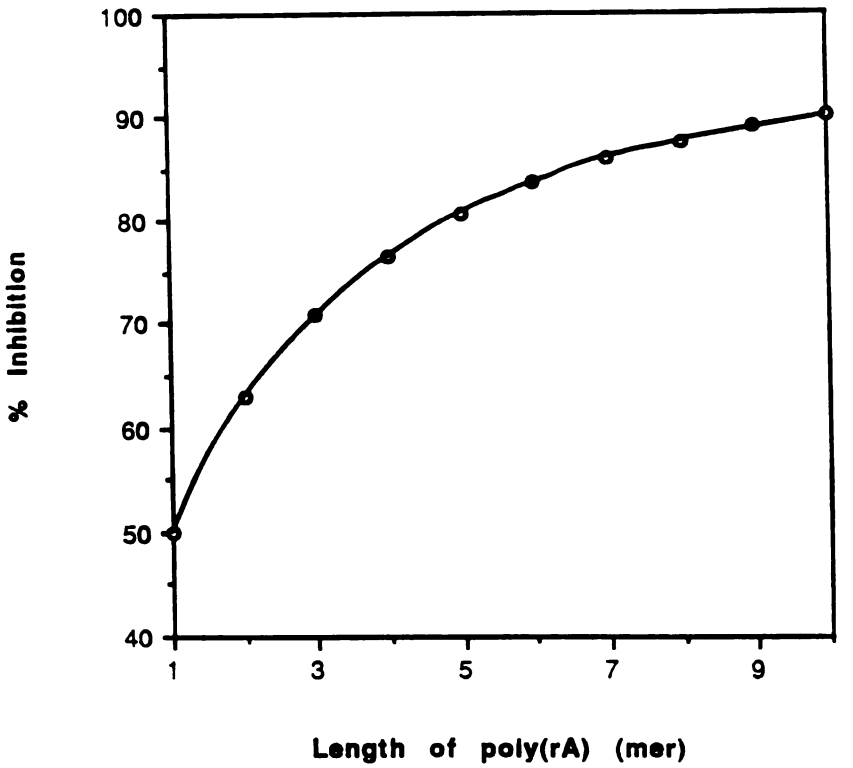


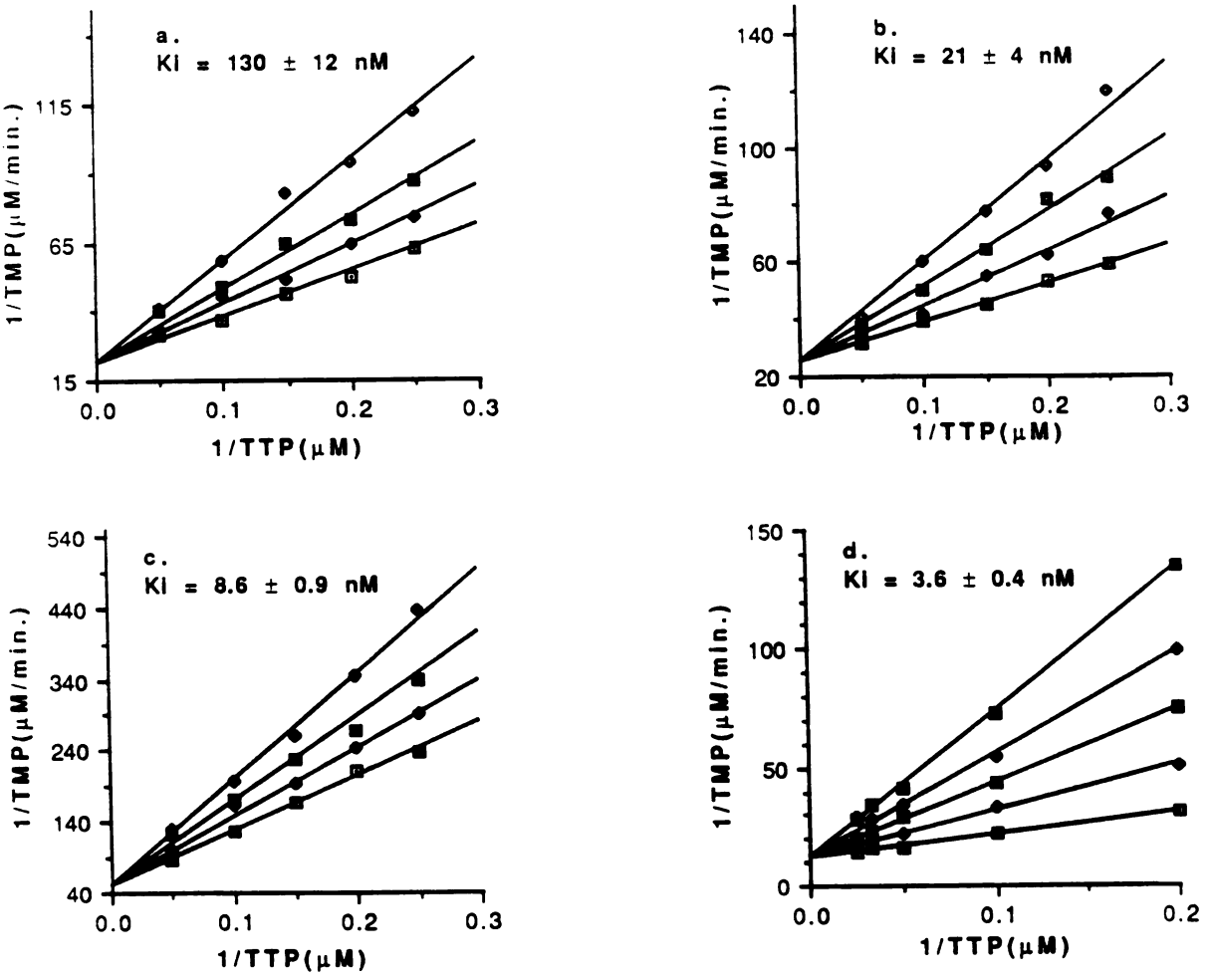
Fig. 5-2. Theoretical plot for the dependence of the inhibition of HIV-1 RT by AZTTP on the length of poly(rA) region of a RNA template.

In order to test our hypothesis, we created a special set of RNA's as templates to study the inhibition of HIV-1 RT by AZTTP. As shown in Fig. 5-3 (e), the only difference among these RNA templates is the number of adenosyl residues following the sequence

complementary to the primer. The double reciprocal plots and K_i values for AZTTP for each template are given in Fig. 5-3 (a-d). The results are fully consistent with our hypothesis. For the template allowing only one TMP moiety to be incorporated into the DNA strand, the observed K_i value for AZTTP is 132 nM. For the template allowing 50 TMP moieties to be incorporated, the observed K_i value for AZTTP drops to 8 nM. In agreement with the previous literature reports (Eriksson et al., 1989), for the template allowing a large number of TMP moieties to be incorporated, i. e., poly(rA):oligo(dT), we obtained an observed K_i value of 4 nM (Fig. 5-3).

VI. Conclusion

Our analysis suggests that AZTTP is not so potent a competitive inhibitor as was previously thought and that AZTTP acts primarily as a DNA chain terminator. This finding is important for the process of discovery of new drugs for the treatment of HIV infection. A common method of evaluating a new drug candidate is to compare it with existing drugs. AZT is currently the only drug that has FDA approval for the treatment of HIV infection, and, if we use the lower, artifactual K_i value for AZTTP as a criterion to search for new drugs, we will lose interest in further investigation of some drug candidates simply because of the "large K_i value difference" from AZTTP. We conclude that homopolynucleotides should not be used as a template to study chain termination inhibitors since the inhibition parameter obtained is less meaningful and cannot be usefully compared with other classes of inhibitors. A system allowing only one nucleotide incorporation can lead to a meaningful K_i value, but this K_i value does not reflect the processive events in



e.

RNA 3'-CCCCUAGGAGAUCUCAGCUGG (A)n CGUCCGUACGUUCGAAGGCCAGAGGG
DNA 5'-GGGGATCCTCTAGAGTCGACC

n = 1 $r(A)_1$:d_{21}mer

n = 10 $r(A)_{10}$:d_{21}mer

n = 50 $r(A)_{50}$:d_{21}mer

Fig. 5-3. Inhibition of HIV-1 RT by AZTTP with different RNA's as templates.

a. $r(A)_1$:d_{21}mer as a template-primer. The concentration of AZTTP: 0 to 0.2 μM.

b. $r(A)_{10}$:d_{21}mer as a template-primer. The concentration of AZTTP: 0 to 0.04 μM.

c. $r(A)_{50}$:d_{21}mer as a template-primer. The concentration of AZTTP: 0 to 0.01 μM.

d. poly(rA):$(dT)_{12-18}$ as a template-primer. The concentration of AZTTP: 0 to 0.008 μM.

e. Structures of the sequence defined template-primers.

the polymerization. Furthermore, the physical meaning of the classical K_i value for a chain termination inhibitor is also questionable since it reflects a more complex set of events than are presumed in the classical definition of a K_i value for competitive inhibition. Unlike the classical K_i value, which only reflects the binding potential of an inhibitor to an enzyme, the K_i value obtained for a chain terminator can reflect a combination of substrate binding effects, intermediate binding effects and the effects of product release. Thus, a new system should be considered to examine potential chain terminators.

6

New Thymidine Triphosphate Analogue Inhibitors of Human Immunodeficiency Virus-1 Reverse Transcriptase

I. Abstract

Several imidotriphosphate analogues of thymidine have been synthesized for the first time and have proved to be effective inhibitors of human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT). When the α,β-bridging oxygen of TTP or AZTTP were replaced by a nitrogen, the resulting analogues are no longer the substrates but competitive inhibitors for HIV-1 RT. The most potent is thymidine 5'-[α,β-imido]triphosphate (TMPNPP), which has a K_i value of 2.4 μM, thus inhibiting HIV-1 RT 100 fold more potently than it inhibits DNA polymerase I large fragment (Klenow). In contrast, substitution at the β,γ-bridging oxygen by nitrogen does not block the enzymatic cleavage of the adjacent α,β-phosphate linkage. 3'-Azidothymidine 5'-[β,γ-imido]triphosphate (AZTMPPNP), the 5'-[β,γ-imido]triphosphate analogue of AZTTP, turns out to be both a substrate and a potent inhibitor for HIV-1 RT. Further nitrogen substitution of the bridge oxygen in the phosphate chain decreases the inhibitory potency by approximately 10 fold, as in the case of thymidine 5'-[α,β:β,γ-diimido]triphosphate (TMPNPNP).

II. Introduction

Human immunodeficiency virus 1 reverse transcriptase (HIV-1 RT) plays an important role in the life cycle of the virus and has been a major target for the design of drug to combat AIDS (Hirsch, 1990; Mitsuya & Broder, 1986; Sandstrom et al., 1985;

Pauwels et al, 1990; Nakane & Ono, 1990; Merluzzi et al., 1990). One important class of HIV-1 RT inhibitors is that of nucleoside analogues like AZT, which are considered to be prodrugs for the treatment of HIV infection (Clercq, 1989; Mitsuya et al., 1985; Furman et al., 1986). These analogues are converted into their triphosphate forms by cellular enzymes, and the triphosphate forms are then recognized by HIV-1 RT as substrates and the corresponding nucleoside monophosphate moieties are incorporated into DNA chain. Since these analogues lack the 3'-hydroxyl group, their incorporation leads to DNA chain termination.

The potency of a chain terminator depends on the rate at which it is incorporated into a DNA chain. This rate is principally governed by two factors, the affinity of a chain terminator for the enzyme and its turnover rate. Understanding structure-activity relationships of nucleotides is important for the design of new HIV-1 RT inhibitors. Although many HIV-1 RT inhibitors have been discovered and studied, little has been reported concerning the influence of various substitutions on nucleotide analogues. The kinetic parameters of these inhibitors most generally reported in the literature are their K_i values and IC_{50} values. Unlike the classical K_i value, which is a measure of the affinity of an inhibitor to the enzyme, the K_i value for a chain terminator reflects the interactions of the enzyme with the inhibitor itself, those of its intermediates and those of the products that it generates. As we pointed out in Chapter 5, K_i values are generally obtained using different homopolynucleotides as templates, and as the magnitude is template-dependent, they cannot been used to compare the inhibitory potencies of the chain terminators to different classes of inhibitors (e. g., simple competitive binding inhibitors).

In contrast to K_i values found for chain terminators, K_i values for the corresponding nonhydrolyzable analogues can be used to compare their affinity to the enzyme, and the effects of nucleoside substituents on their affinity can be obtained from the comparison of their K_i values. We have designed and synthesized a series of novel

nonhydrolyzable NTP analogues, and the relationship between their substitution and affinity for HIV-1 RT has been revealed from kinetic studies.

III. Experimental

A. Materials and Methods

^{31}P NMR spectra were obtained at 79.5 MHz on a Nicolet NTCFT-1180 NMR spectrometer. A sweep width of 4000 Hz, a probe temperature of 25°C, and ^{1}H broad-band decoupling were used in ^{31}P NMR measurements. Chemical shifts were determined relative to 85% H_3PO_4 with positive shifts being downfield from the reference. High- and low- resolution LSIMS spectra were recorded on a Kratos MS-50 mass spectrometer and a negative ion probe was used to measure the mass of the M-1 peak.

PEI- cellulose plates were obtained from J. T. Baker Co. Other chemicals were from Aldrich. Poly(rA), $(dT)_{14}$, DNA polymerase I large fragment (Klenow), polynucleotide kinase and deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP) were from Pharmacia; HIV-1 reverse transcriptase-p66 was prepared as described previously (Rowley et al., 1990).

B. Synthesis

Tetrasodium imidodiphosphate, trichloro((dichlorophosphoryl)-imino)phosphorane and **(P,P-dichloro-N-(dichlorophosphinyl)phosphinimyl)-phosphorimidic trichloride** were prepared as described previously (Ma et al., 1988; Ma et al., 1989; Ma et al., 1990). **Dideoxythymidine** was synthesized from thymidine by the methods reported in the literature (Robins & Robins, 1967). They were used as the precursors for the synthesis of thymidine triphosphate analogues.

AZTMPNPP and **TMPNPP** were synthesized and characterized as described in chapter 5.

a. Dideoxythymidine 5'-[α,β-imido]diphosphate (ddTMPNP)

ddTMPNP was synthesized from dideoxythymidine (0.5 mmol) using the methods for TMPNPP synthesis (see Chapter 5), and the yield was 38% of theoretical. It gave a single spot on PEI-TLC with a R_f value comparable to that of TDP (0.3 M TEAB buffer as an eluent, $R_f = 0.55$). LSIMS gave the expected molecular weight (M-1 = 384).

b. Dideoxythymidine 5'-[α,β-imido]triphosphate (ddTMPNPP)

ddTMPNP (0.1 mmol) was then used to synthesize ddTMPNPP, and a yield of 25% was obtained by the same procedure used for the TMPNPP synthesis. ddTMPNPP was characterized by TLC, UV, NMR and MS. It showed a single spot on PEI-TLC with the same R_f value as TTP (0.4 M TEAB as an eluent, $R_f = 0.4$). UV showed maximum absorption at 266 nm. ^{31}P NMR (D_2O, pD = 7.5, ^1H broadband decoupling on): δ 2.69 (1 P, s, α-P), -5.7 (1 P, d, J = 19.8 Hz, γ-P), -8.52 (1 P, d, J = 20 Hz, β-P). High resolution LSIMS (monosodium mass, M-1): calculated for $C_{10}H_{16}N_3O_{12}P_3Na$: 485.9845; found: 485.9845.

c. Thymidine 5'-[α,β:β,γ-diimido]triphosphate (TMPNPNP)

TMPNPNP was synthesized using a different method from that used for the AMPNPNP synthesis(Ma et al., 1990). Dried thymidine (3 mmol) was dissolved in 3 mL of triethyl phosphate and the solution was cooled to -15°C. (P,P-dichloro-N-(dichlorophosphinyl)phosphinimyl)-phosphorimidic trichloride in triethyl phosphate (3

mL) was added to the mixture dropwise with stirring. After 1 hour, the reaction was quenched with 15 mL of 0.1 N HCl and kept at 0°C for 20 min. The mixture was then neutralized by 1 N NaOH and extracted with 3 aliquots of 25 mL ethyl acetate. The water layer was evaporated under vacuum until 5 mL remained, and the residue was loaded onto a AG^{R}MP-1 column. TMPNPNP was purified and converted to sodium salts in the same way as described in the TMPNPP synthesis. A white powder was obtained(0.8 mmol, 26% yield). The UV spectrum and TLC behavior are the same as for TMPNPP. ^{31}P NMR (D_2O, pD = 10, ^{1}H broadband decoupling on): δ 3.93 (1 P, d, J = 7 Hz,), 2.85 (1 P, d, J = 4 Hz),1.01 (1 P, d, J = 4 Hz). High resolution LSIMS (disodium mass, M-1): calculated for $C_{10}H_{16}N_4O_{12}P_3Na_2$: 522.9774; found: 522.9766.

d. 3'-Azido-3'-deoxythymidine 5'-triphosphate (AZTTP) and 3'-Azido-3'-deoxythymidine 5'-[β,γ-imido]triphosphate (AZTMPPNP)

AZTTP) and AZTMPPNP were prepared by the method of Vrang et al.(1987). AZT (3 mmol) was dissolved in 5 mL triethyl phosphate and cooled to 0°C. Oxyphosphoryl trichloride (3 mmol) was added to the mixture with stirring, and the reaction was kept at 0°C for 1 hour. Mono(tributylammonium) salts of diphosphate analogues (pyrophosphate for AZTTP and imidodiphosphate for AZTMPPNP, 6 mmol) and 6 mmol of tributylamine were added to the mixture, and the solution was kept at 0°C for another 10 minutes. The reaction was quenched with 20 mL of 0.1 N NaOH, and the residue was further treated as described for the TMPNPP synthesis. The yields are 47% for AZTTP and 25% for AZTMPPNP, respectively. Both compounds showed the same behavior on PEI-TLC and gave the same UV spectra as TMPNPP. AZTTP was characterized by ^{31}P NMR and LSIMS, and the data were fully consistent with the structure (Vrang et al., 1987). AZTMPPNP was characterized in the same manner. ^{31}P NMR (D_2O, pD = 10, ^{1}H broadband decoupling on) δ -8.21 (1 P, d, J = 20 Hz, α-P), -4.95 (1 P, dd, J = 20 Hz, 4

Hz, β–P), 2.25 (1 P, d, J = 20 Hz, γ-P). High resolution LSIMS (monosodium mass, M-1): calculated for $C_{10}H_{15}N_6O_{12}P_3Na$: 52526.9877; found: 526.9859.

AZTMPPNP was further treated with hexokinase to remove possible AZTTP contamination [This procedure is based on our finding that AZTTP is a substrate for hexokinase but that AZTMPPNP is not]. The sodium salt of AZTMPPNP (0.5 μmol) was dissolved in 2 mL of buffer (pH 8) containing: 200 mM glycine, 12 mM $MgCl_2$, 15 mM NADP, 100 mM glucose, 100 units of glucose-6-phosphate dehydrogenase and 200 units of hexokinase. The mixture was incubated at 25°C for 12 hours and then loaded onto a DEAE-Sephadex A-25 column (1 x 20 cm) and eluted with 100 mL of 0 - 0.5 M linear gradient of TEA-HCO_3^- (pH 8.5). The appropriate fractions were pooled, and the TEAB buffer was removed under high vaccum. AZTMPPNP was then converted to its sodium salt by passing the residue through a Sp-Sephadex column (Na^+ form) and the desired fractions were lyophilized to dryness. The AZTMPPNP obtained was ready for kinetic studies.

C. Enzyme Kinetics

a. HIV-1 RT inhibition assay

The assay mixture (50 μl) for the kinetic study contained: 50 mM Tris-HCl (pH 8.0), 6 mM MgCl, 8 mM DTT, 80 mM KCl, 0.4 μM poly(rA)-oligo(dT), 60 ng/ml of HIV-1 RT (P66) , 0.4 μM poly(rA)oligo(dT), different inhibitors,and variable amounts of ^{3}H-TTP (4 to 20 μM, 0.2-1 μCi/20 μl). After incubation at 37°C for 30 min., the mixtures were quenched by 5 μl of 0.5 M EDTA, and the template-primers were collected on DE-81 paper (Furman et al., 1979). The incorporation rate of TMP moieties was measured by counting ^{3}H on the DE-81 paper, and the K_i values were calculated from double reciprocal plots.

b. DNA polymerase I inhibition assay

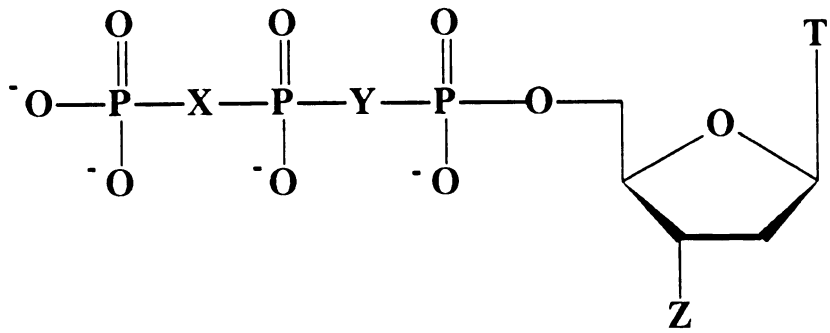
The assay mixture (50 µl) contained: 50 mM Tris-HCl (pH 7.5), 6 mM MgCl, 8 mM DTT, 80 mM KCl, 0.4 µM poly(dA):oligo(dT), 20 µM ^{3}H TTP (1 µCi/20 µl), 20 units/ml of DNA polymerase I large fragment (Klenow) and variable amounts of either TMPNPP or AZTMPPNP. The incorporation of TMP moieties was measured in the same way as described in HIV-1 RT inhibition assay.

c.Primer extention assay

$(dT)_{14}$ (Pharmacia) was 5'-labeled with ^{32}P by polynucleotide kinase according to the method recommended by the manufacturer (Pharmacia). The 5'-^{32}P$(dT)_{14}$ was then annealed with equimolar amounts of poly(rA), and the resulting template-primer was used for the primer extention assay. The reaction mixture contained: 50 mM Tris-HCl (pH 8.0), 6 mM MgCl, 8 mM DTT, 80 mM KCl, 0.4 µM poly(rA)oligo(dT), 0.1 µM poly(rA)-5'-^{32}P$(dT)_{14}$, 20 µM TTP (or TTP analogues) , 0.3 µg/ml HIV-1 RT(P66). After incubation at 37°C for 1 hr., the reaction was quenched with EDTA, and the products were identified by 20% polyacrylamide/7M urea gel.

IV. Results and Discussion

A. Synthesis of TTP analogues



Name	*X*	*Y*	*Z*
TMPNPP	*O*	*N*	*OH*
AZTMPNPP	*O*	*N*	N_3
ddTMPNPP	*O*	*N*	*H*
TMPNPNP	*N*	*N*	*OH*
AZTMPPNP	*N*	*O*	N_3

Fig. 6-1. Structures of TTP Analogues

Previous syntheses of 5'-[α,β-imido]triphosphates of nucleosides involved the reactions of nucleoside 5'-[α,β-imido]diphosphates with phosphocreatine catalyzed by creatine kinase (Ma et al. 1988, 1989, 1990). Though TDP is a good substrate for several kinases, such as creatine kinase, pyruvate kinase and arginine kinase (data not shown), TMPNP is not a substrate for these kinases. We utilized the activation of the phosphate moiety of NMPNP by carbonyl diimidazole as an alternative way to incorporate the terminal phosphate. Although we also tried other chemical methods, such as activation of inorganic phosphate with carbonyl diimidazole, only the procedures described here gave detectable products.

The only existing nucleoside 5'-[α,β-diimido]triphosphate(NMPNPNP) is AMPNPNP, which was synthesized by reaction of 5'-tosyl adenosine with tetrabutylamonium salts of diimidotriphosphate (Ma et al., 1990). The reaction also

generated another product, $AMP(NP)_2$, which is very difficult to separate from AMPNPNP. We have successfully synthesized TMPNPNP by the reaction of thymidine with (P,P-dichloro-N-(dichlorophosphinyl)phosphinimyl)-phosphorimidic trichloride in triethyl phosphate followed by acidic water hydrolysis. No branched products were detected from the reaction mixture in this case, and the products were readily purified. The unique step of this reaction is the acidic hydrolysis. The classical hydrolysis of phosphazine derivatives of nucleosides are carried out in basic solutions, which does not work for the synthesis of TMPNPNP.

AZTMPPNP is a potent inhibitor of HIV-1 RT (Table 6-1). Since this compound is similar to AZTTP, it is possible that the inhibition could result from AZTTP-contamination during synthesis. We have developed a novel method to eliminate possible contamination by AZTTP. We found that AZTTP is a good substrate for hexokinase but that AZTMPPNP is not (data not shown). Using this enzymatic procedure, AZTTP can be quantitatively converted into AZTDP. The inhibitory potency of AZTMPPNP was tested before and after hexokinase treatment and showed no significant difference (data not shown).

B. Kinetic studies of TTP analogues with modified polyphosphate chains.

Although nucleoside triphosphate analogues have been studied extensively as HIV-1 RT inhibitors, no systematic investigation has been performed on polyphosphate chain modification. We have studied the effects of the bridge oxygen substitution by nitrogen on the enzymatic kinetics. Since PNP bond resembles POP bond in bond angle, bond length and other properties but resistant to enzymatic cleavage (Larsen et al., 1969), we reasoned that the α,β-imidotriphosphate analogues of nucleosides would be the best nonhydrolyzable analogues for kinetic studies. When the α,β-bridging oxygen of TTP was replaced by nitrogen, the resulting compound, TMPNPP, is no longer a substrate for

HIV-1 RT (see Fig. 6-2, lane 5). Instead, it is a good competitive inhibitor for the enzyme with a K_i value of 2.4 μM (Table 6-1).

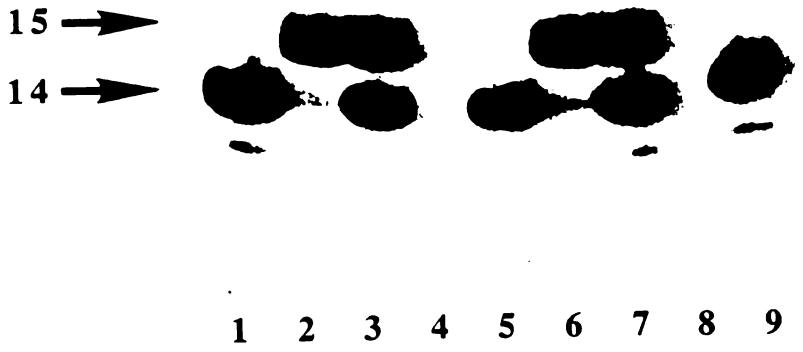


Fig. 6-2. Primer extention assay for TTP analogues

The assays were performed at the conditions described in experimental except the following difference in reactions a to e. a, no TTP or TTP analogues; b, with 20 μM AZTTP; c, with 20 μM AZTMPPNP; d, with 20 μM TTP; e, with 20 μM TMPNPP. The five reactions were quenched after incubation at 37℃ for 1 hours and then were electrophoresized on a 20% polyacrylamide/7 M urea gel. Lane 1, a; lane 2, b; lane 3, a + b; lane 4, d; lane 5, e; lane 6, c; lane 7, c + a; lane 9, a.

AZTMPPNP, the 5'-[β,γ-imido]triphosphate analogue of AZTTP, turned out to be both a substrate for HIV-1 RT and a relatively potent competitive inhibitor for the enzyme (see

Fig. 6-2, lane 6; Table 6-1). These results show that the substitution of the bridging oxygen by nitrogen does not block the cleavage of the adjacent POP bond and the inhibitory potency of AZTMPPNP partially results from DNA chain termination.

Further substitution of the bridge oxygen of TMPNPP by nitrogen decreases the binding to HIV-1 RT by an order of magnitude (Table 6-1). These results parallel those found for AMPNPP and AMPNPNP in their interactions with S-adenosyl methionine synthetase (Ma et al., 1990). In the case of S-adenosyl methionine synthetase, both AMPNPNP and ATP have the same affinity for the enzyme but AMPNPP binds to the enzyme 60-fold more tightly than does ATP. Thus, diimido nucleotide analogues appear generally to bind to enzymes less efficiently than monoimido analogues.

C. Inhibition of HIV-1 RT by TMPNPP analogues with modified ribose moieties

Several nucleotide triphosphate analogues have proven to be inhibitors for HIV-1 RT. Among these are AZTTP and ddTTP, whose K_i values are comparable to each other (Cheng et al., 1987). Since they are DNA chain terminators, the fact that they have the

Table 6-1. Inhibition of HIV-1 RT by TTP analogues

Compounds	Inhibition type	Ki (μM)
TMPNPP	Competitive	2.4 ± 0.1
ddTMPNPP	"	15 ± 0.9
AZTMPNPP	"	22 ± 1.3
AZTMPPNP	"	0.087 ± 0.005
TMPNPNP	"	19.1 ± 1.0

same observed K_i values do not mean that they necessarily have the same affinity to the enzyme. Since nucleoside α,β-imidotriphosphate can bind to, but cannot be cleaved by, HIV-1 RT, their observed K_i values can be used to compare the relative affinities of these analogues for the enzyme. As shown in Table 6-1, TMPNPP, AZTMPNPP and ddTMPNPP are typical competitive inhibitors with Ki values of 2.4, 23, 15 μM, respectively. We conclude that 3'-azido substitution does not increase the affinity of NTP's to the enzyme. As we pointed out previously (Ma, et al., 1991), the reported low K_i value of 4 nM for AZTTP is an artifact of the use of homopolynucleotides as template-primers. These findings are important for the design of new HIV-1 RT inhibitors. We now know that AZTTP binds to the active site of HIV-1 RT weaker than TTP, not stronger as proposed previously (Keder et al., 1990). This infers that it is promising to find new HIV-1 RT inhibitors which will have a better fit to the enzyme than AZTTP since the binding affinity of AZTTP has not been optimized.

d. The imidotriphosphate analogues of TTP are specific inhibitors for HIV-1 RT

As shown in Table 6-2, both TMPNPP and AZTMPPNP are much better inhibitors for HIV-1 RT than for DNA polymerase I large fragment (Klenow). In the case of TMPNPP, the 50% inhibition concentration for HIV-1 RT is at least 200 fold lower than that for DNA polymerase I (Klenow). Since TMPNPP is a nonhydrolyzable substrate inhibitor for HIV-1 RT, this result suggests that TMPNPP binds to the active site of HIV-1 RT much more tightly than to that of DNA polymerase I. Since the reported K_m values for TTP are close to the same for HIV-1 RT and DNA polymerase [K_m = 3.9 μM for DNA polymerase I (Slater et al., 1972); K_m = 5.9 μM for HIV-1 RT (Hizi et al., 1991)], the selective inhibition of HIV-1 RT by TMPNPP could arise from the substitution of imido group. Further investigation, including cocrystallizing TMPNPP with HIV-1 RT and

determining the three-dimensional structure of the resulting complex could give clearer insights regarding these observations.

Table 6-2. Specificity of AZTMPPNP and TMPNPP

	50% Inhibition Concentration (μM)	
Enzyme	TMPNPP	AZTMPPNP
HIV-1 RT	10	0.1
Klenow	4000[a]	2000[a]

[a] *only ~25% inhibition was observed at this concentration.*

Compared with TMPNPP, AZTMPPNP is a better inhibitor of HIV-1 RT, and its inhibition potency towards HIV-1 RT is ~1000-fold greater than for DNA polymerase I. The cause of this specificity is complicated by the fact that AZTMPPNP is also a substrate for HIV-1 RT and DNA polymerase I (data not shown). Apparently, the potent inhibition of HIV-1 RT arises partially from the incorporation of AZTMP moiety into the DNA chain causing chain termination. The specificity might result from the tight binding to HIV-1 RT, from the faster turnover of AZTMPPNP to products in the HIV-1 RT assay system, or simply from the 3' to 5' proof-reading activity of the Klenow fragment which abolishes termination.

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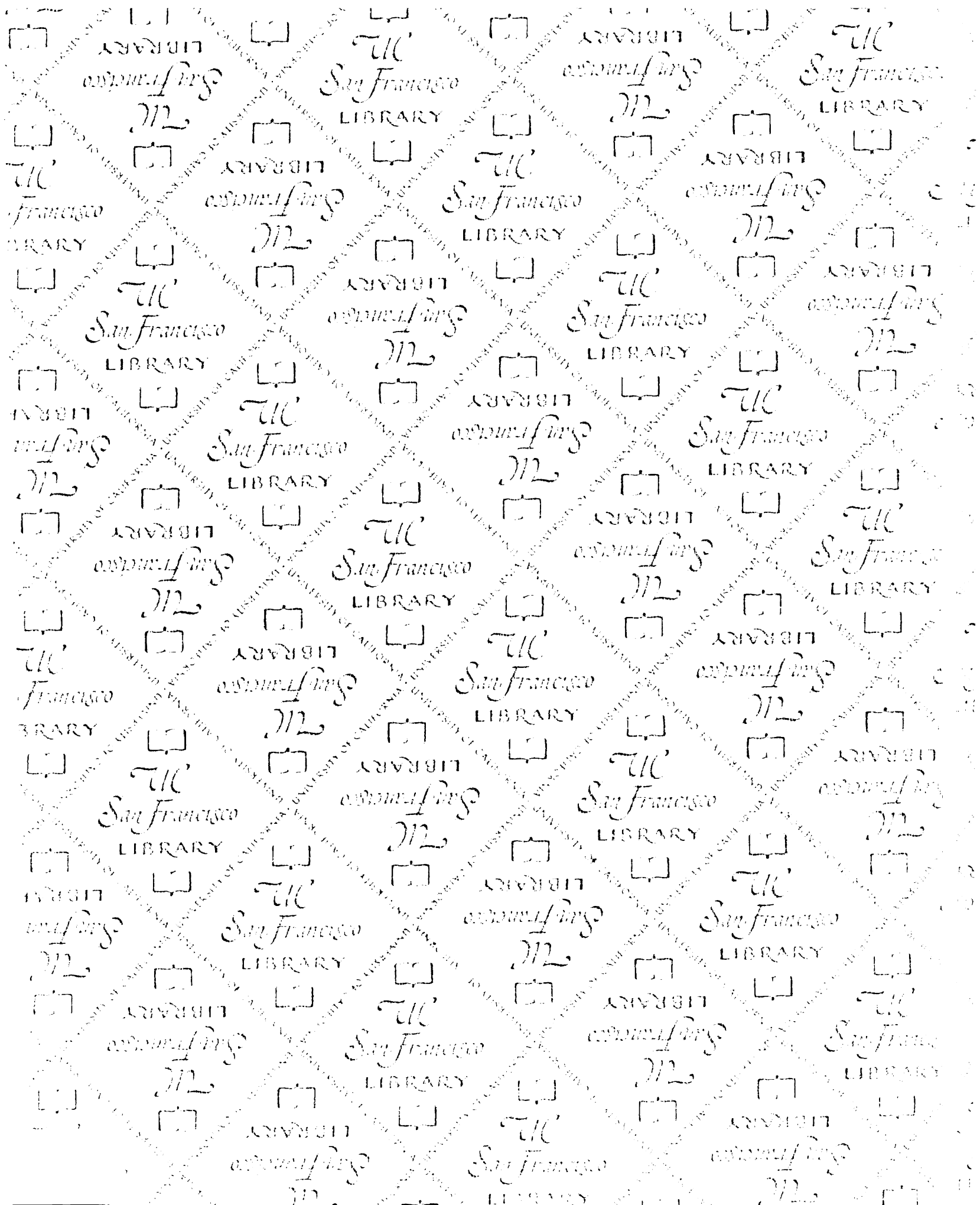
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