ACTIVE SITE STUDIES OF TWO TARGET ENZYMES:
REVERSE TRANSCRIPTASE AND ASPARTATE TRANSCARBAMOYLASE

by

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ABSTRACT

Two target enzymes for chemotherapy were investigated:
by probing their active sites with various methods. The
reaction catalyzed by Aspartate Transcarbamoylase from
Escherichia coli is thought to involve a tetrahedral
transition state. Several bi-substrate analogs with a
charged tetrahedral moiety were synthesized and tested for
inhibition. The results suggest that the enzyme exhibits no
preference for a tetrahedral over a trigonal moiety which
casts some doubt as to the actual reaction mechanism. The
carbamoyl region of the active site of the enzyme was
further probed with various anionic inhibitors to
investigate the structural basis for the strong inhibition
observed with phosphonoformate and carboxyldiphosphonate.

A second target enzyme, reverse transcriptase that is also
effectively inhibited by these two anionic compounds was
studied. The active site of this enzyme was probed with a
variety of anionic ligands to determine the structural
features necessary for inhibition. Phosphonoformate
prevailed as the most effective inhibitor of three different
reverse transcriptases. Combined inhibitor studies with the
enzyme from Human Immunodeficiency Virus Type-1 were
performed to further investigate the mode of binding of PPA
with the active site. Finally, the interaction of the metal
chelator ortho-phenanthroline with the enzyme was investigated. A kinetic analysis and protection/inactivation studies were performed to probe the site of metal chelation and its proximity to the binding sites for various active site ligands.

ACKNOWLEDGEMENTS

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ABBREVIATIONS
AIDS acquired immunodeficiency syndrome
AVM avian myeloblastosis virus
ATCase aspartate transcarbamylase
Amp aspartate
ATP aconitase triphosphate
Bis enzyme reaction involving two substrates and two products
Cp catalytic subunit of aspartate transcarbamylase from Escherichia coli
CpCu aspartate transcarbamylase
cpm counts per minute
δ NMR chemical shift (parts per million) downfield from tetramethylsilane
D2O deuterium oxide
DTT dithiothreitol
HPPES N-2-hydroxyethylpiperazine-N’-2-ethanesulfonate
His histidine
HIV human immunodeficiency virus type-1
Hs hours
IC50 concentration of inhibitor required to cause 50% inhibition of enzyme at a specific substrate concentration
Kcat the turnover number of the enzyme; the maximum number of catalytic processes the enzyme can catalyze per unit time
Km inhibition constant. Equilibrium constant for E + I → EI
Km Michaelis constant
Lys lysine
MLV Moloney murine leukemia virus
mp melting point
NTP nucleoside triphosphate
NMR nuclear magnetic resonance
1,10 (orthon)-phenoanthroline
OP n-phosphonomethanol-N-aspartate
Pol I Escherichia coli DNA polymerase I
pKa negative logarithm of acid dissociation constant
Q2 regulatory subunit of aspartate transcarbamylase from Escherichia coli
reverse transcriptase
RT reverse transcriptase
Thi threonine
TLC thin layer chromatography
tHd thyamine diphosphate
tHm thyamine monophosphate
tris (tris(hydroxymethyl))aminomethane
UV ultraviolet
1. INTRODUCTION

Most chemical reactions of living organisms would occur extremely slowly were it not for the presence of protein catalysts, or enzymes. These catalysts are critical not only for the energy-coupling processes of living organisms but for the synthesis of biomolecules required for their maintenance, growth and multiplication. Like other catalysts they lower the free energies of activation of the reactions they catalyze. However, they display a remarkably high efficiency and an extraordinary substrate specificity not found with other catalysts. For this reason, a large research effort has been directed towards understanding these two features of enzyme action since the discovery of enzymes 150 years ago.

Compounds which inhibit specific enzymes have long been used as powerful tools in various fields of investigation. Specific enzyme inhibitors have been used to investigate the sequence of reactions that occur in multiple enzyme systems involved in linear or cyclic metabolic pathways. Two classic examples of this application is the mapping of the glycolytic pathway by Lehmann and Meyerhof (Lehmann, 1931; Meyerhof & Boyland, 1931), and the identification of specific loci in the electron transport chain (Keilin & Hartree, 1955; Chance

\[ \text{\textcopyright Williams, 1956.} \] Pathologists have used inhibitors to establish a metabolic blockade in experimental animals that produce syndromes similar to certain disease states, and have thereby learned more about the specific disease process (Webb, 1966). For example, the chronic administration of alloxan produces progressive pathological changes similar to diabetes, and the administration of monoamine oxidase inhibitors has been used to produce abnormal mental states similar to those observed in man.

On the biochemical level, the interaction of an inhibitor with a purified enzyme has been studied to characterize the protein and to help understand its catalytic mechanism. Another worthwhile field of research involving enzyme inhibitors is the treatment of pathological tissues with a known metabolic aberration. Treatment with substances that block a specific reaction along the metabolic pathway of interest are sometimes able to restore cells to normal and can therefore be used in the treatment of the associated condition (Webb, 1966). When the disease state involves a foreign organism, the differences between the host tissue and the invading organism can be determined and used to design inhibitors that block a vulnerable reaction in the infectious agent. If administration of the inhibitor causes a significant imbalance in the host cells it is labelled toxic, but if it mainly alters the invading organism or the
pathological tissues it is considered a drug. This type of investigation has established a close relationship between pharmacology and the study of enzyme inhibitors (Wass, 1966; Abeles, 1978).

1.1 Target Enzymes

In order for a specific inhibitor to be considered as a putative drug, its target enzyme must be required to propagate the diseased state. It must also be specifically situated within the metabolic pathways of a cell such that its inhibition mainly affects the desired process. An example of this type of target enzyme is aspartate transcarbamoylase (ATCase) which is the subject of part of this thesis. It plays an important role in cellular proliferation because it provides the precursors for the biosynthesis of nucleic acids and RNA. ATCase catalyzes the first unique step in pyrimidine synthesis where carbamoyl phosphate reacts with aspartate to form N-carbamoyl-L-aspartate. The enzyme is a suitable target for the chemotherapy of cancer, since it is found in elevated levels in rapidly proliferating tissues including several tumors (Kilford, 1976; Medani et al., 1987). A blockage of this enzyme produces few side effects in a cell since only the substrates carbamoyl phosphate and aspartate and the substrates of the preceding enzyme in the pyrimidine biosynthetic pathway, bicarbonate and glutamine would accumulate as a consequence of inhibition at this early point in the pyrimidine synthetic pathway and none of these compounds is likely to be toxic. In contrast, modified nucleosides such as 5-fluorouracil and 6-azauridine inhibit at later stages in the nucleotide synthetic pathways and can have deleterious side effects due to accumulation of toxic precursors and incorporation of the modified nucleosides into RNA.

When the diseased state is due to a viral or bacterial infection, the essential enzymes of the invading organism represent putative targets for intervention. This is particularly true if one of these enzymes either catalyzes a unique reaction required for the persistence of the organism or displays unique characteristics that can be exploited for the development of specific inhibitors with minimal effects on the corresponding host cell enzymes. A well-known example of this is the enhanced sensitivity of viral DNA polymerases to the inhibitory effects of certain nucleoside analogs compared to host cell DNA polymerases (Smoler et al., 1971). This is due to the finding that the viral DNA polymerases have significantly less stringent structural requirements for the binding of nucleoside triphosphate (NTP) analogs to the substrate binding site compared to host cell DNA polymerases. Another viral enzyme which displays distinctive characteristics compared to its eukaryotic counterparts is the protease from the human immunodeficiency virus type-1 (HIV). The enzyme is similar to pepsin and other aspartyl proteases in that it has two active site carboxyl residues involved in catalysis (Saelmier et al., 1988). However, the HIV enzyme is unusual in that it has an active site with a twofold axis of symmetry and is therefore effectively inhibited by specifically designed symmetrical compounds (Ericksen et al., 1990).

1.2 Chemical Catalysis

Enzymes are able to speed up the reactions they catalyze by factors of up to $10^9$ (Frick et al., 1987). There are several important features of enzymes that allow them to catalyze with such efficiency, and these are best illustrated by comparison with those of uncatalyzed reactions.

1.2.1 Transition State Theory

The transition state theory relates the difference in Gibbs energy between the transition state, the most unstable species on the reaction pathway and the ground state or reactants to the rate of a reaction (Polzer & Wigner, 1933). The transition state occurs at the peaks of the energy profile of a reaction, when chemical bonds are in the process of being formed and broken (Figure 1). Since the transition state involves the breaking of existing bonds and the formation of new bonds, the energy profile of the reaction will be characterized by the energy needed to break bonds (activation energy) and the energy released by forming the new bonds (reaction energy). The reaction energy is the net energy change in the reaction, while the activation energy is the energy needed to overcome the energy barrier of the transition state. The energy profile of the reaction can be represented as a series of energy levels, with the reactants at the lowest energy level and the products at the highest energy level. The activation energy is the energy required to overcome the energy barrier of the transition state, and is the energy difference between the energy of the reactants and the energy of the transition state.
and ground states of a reaction are in thermodynamic equilibrium, the concentration of the transition state can be obtained from the difference in their energies. By multiplying the concentration of the transition state by its rate constant for decomposition, the rate of a reaction may be obtained. Since at a given temperature all transition states decompose at the same rate, the rate of reaction is solely dependent upon the free energy change associated with the formation of the transition state ($E_{\Delta}$).

Enzymes are able to accelerate reactions by stabilizing the transition state, thereby lowering the free energy required to attain the transition state. The reaction coordinate diagram for a catalyzed and uncatalyzed reaction are shown in Figure 1. The troughs represent unstable intermediates formed during the reaction. According to the Hammond postulate (Hammond, 1955) these unstable intermediates will more closely resemble the transition state than do the substrates or products. Therefore characterization of an intermediate can assist in the prediction of the transition state.

1.2.2 RATE ENHANCEMENT FEATURES

In contrast to non-protein catalysts (such as OH, NO, and metal ions) each enzyme catalyzes a small number of reactions, frequently only one. The ability of an enzyme to catalyze one specific reaction and essentially no others is perhaps its most significant property. Most enzymes catalyze the same reaction with a small number of structurally related compounds because they have well-defined binding sites for their substrates. This promotes the formation of an enzyme-substrate complex in an orientation poised for catalysis. In this complex, the catalyst (enzyme) and the substrate are considered to be part of the same molecule before catalysis occurs. The loss of rotational and translational entropies of the substrate molecule associated with binding is paid for by the substrate binding energy of the enzyme. The formation of the enzyme-substrate complex increases the effective concentration of the reactants because the reaction essentially becomes an intramolecular one. This results in an enormous rate enhancement compared to the analogous intermolecular reaction.

Another important feature of enzymes that allow them to greatly accelerate the rate of a reaction is the complementarity to the transition state. Part of the total binding energy of the enzyme is used for binding the substrate(s) while the remainder is applied to the transition state. This causes a destabilization of the bound substrates by electrostatic or geometric strain which promotes the formation of the transition state (Wolffenden, 1976). Since an enzyme will generally display the highest affinity for the transition state of the reaction it catalyzes, knowledge about the mechanism of the enzyme of interest is therefore of great utility when designing highly potent and specific inhibitors.

In summary, two of the most important factors contributing to the rate enhancement by enzymes is the loss of entropy that occurs upon binding the substrates and prior to the catalytic steps, and the transition state complementarity of the active site.

1.3 INHIBITORS

The broadest definition of an enzyme inhibitor is any substance which reduces the rate of an enzyme reaction. There are two major classes of inhibitors, each of which can be used to study the nature of an enzyme.

1.3.1 IRREVERSIBLE INHIBITORS

Irreversible inhibitors are able to permanently inactivate enzymes by covalent modification. The compounds can be classified as 1) affinity labels or active-site directed reagents and 2) suicide inhibitors. The first type of inactivation resembles the substrate to some degree, and contains a reactive group. Affinity labels bind preferentially but not solely to the substrate binding site of the enzyme by non-covalent interactions. The functional group of the affinity label then reacts with an active site residue(s) to covalently modify the enzyme. An example of affinity labeling is the identification of active site lysine residues involved in the binding of the substrate NTP's in DNA polymerases with the use of pyridined phosphates (Bass & Mudak, 1987). These reagents have helped to map out the active sites of enzymes by the specific labeling and identification of important residues within the binding regions of an active site. However, their functional groups react readily with other molecules and therefore these reagents generally lack the desired specificity to be of therapeutic interest.

Suicide inhibitors, or mechanism-based inactivators have much more potential as specific and potent irreversible inhibitors of target enzymes. These compounds bind in the same fashion as substrates but contain dormant reactive functional groups which are activated at some point along the catalytic pathway of the enzyme (Ables & Maycock, 1976). Since the inactivation is mechanism-based and essentially no enzymes have exactly the same mechanism, there is an extremely high degree of specificity involved. The rate of inactivation produced by suicide inhibitors under specific conditions depends on the dissociation constant for the compound and the rate that the enzyme converts it to a reactive species. From a pharmacological standpoint, these
compounds are often selective and long lasting drugs because they cause permanent inhibition of only their target enzyme. An example of this type of compound is trianloylproprine, a suicide inhibitor of monoamine oxidase used in the treatment of depression (Halleren & Krivin, 1968). However, one limitation to the development of suicide inhibitors is that it requires some knowledge of the structure of the active site and the catalytic mechanism of the enzyme.

1.3.2 REVERSIBLE INHIBITORS

Enzymes are also reversibly inhibited by the non-covalent binding of compounds. There are three types of reversible inhibitors according to their mode of inhibition of their target enzyme.

Competitive inhibitors bind to at least part of the substrate binding region of the active site and thereby prevent substrate binding. This type of inhibition can be overcome by increasing the substrate concentration, since the apparent $K_{m}$ for the substrate is altered but not the maximum velocity ($V_{max}$) of the enzyme. Noncompetitive inhibition occurs when the substrate and inhibitor can bind simultaneously to the enzyme and therefore interact with different sites. The inhibition lowers the catalytic efficiency of the enzyme but does not affect the $K_{m}$ of the substrate. An uncompetitive inhibitor on the other hand, will bind only to the enzyme-substrate complex but not to the free enzyme and the resulting ternary complex is inactive.

1.4 MULTIPLE INHIBITOR STUDIES

The characterization of both the type and level of inhibition displayed by various ligands is one way of elucidating the nature of the binding sites of a specific enzyme. However, further information can be obtained by investigating the simultaneous effects of two inhibitors on an enzyme. The spatial relationship for the binding sites of two structurally different inhibitors can be inferred from this type of study. Kinetic analysis of such inhibition by various methods including that of Yonetani and Theorell (Yonetani & Theorell, 1964) can be used to measure the degree of interaction between two inhibitors. This involves varying the concentration of one inhibitor ($I_1$) at different fixed concentrations of a second inhibitor ($I_2$). The following equation of Yonetani & Theorell relates the reaction velocity in the presence of two inhibitors to their concentration and inhibition constants:

$$v = \frac{v_i}{1 + K_{i1} + \frac{K_{i2}}{V_i} + \frac{K_{12}}{V_i K_{i2}} \left(1 + \frac{K_{i1}}{V_i} \right)^{-1} \left(1 + \frac{K_{i2}}{V_i} \right)^{-1} \frac{v_i}{v_{max}} + \frac{I_1}{V_i}}$$

where $v_i$ and $v_i$ are the concentrations of inhibitor 1 and 2 respectively, $v_i$ is the velocity in the presence of both inhibitors, $K_{i1}$ and $K_{i2}$ are dissociation constants for the enzyme complexed with inhibitor 1 and 2 respectively, and $a$ is an interaction constant between $I_1$ and $I_2$ in the $I_1$,$I_2$ complex.

According to Yonetani and Theorell, plotting $v/v_i$ versus $I_1$ will yield a family of lines, one for each different concentration of the second inhibitor. From the point of intersection of these lines the interaction constant ($a$) can be determined which is a quantitative measure of the interaction between two inhibitors at the active site of the enzyme. Yonetani reported that if $0 < a < 1$ the compounds bind synergistically, or the binding of one inhibitor to the enzyme enhances its affinity for the second one (Yonetani & Theorell, 1964). An interaction constant of $a$ indicates that there are separate and non-interacting binding sites for the two compounds. A large value for $a$ shows that there is antagonism between the two inhibitors, and therefore their binding sites overlap either in a kinetic or physical mode.

1.5 TWO TARGET ENZYMES: COMMON INHIBITORS

The body of this thesis is divided into two separate sections, involving active sites investigations of two therapeutically important enzymes, ATCase and reverse transcriptase (RT). ATCase is a catalytic enzyme in the pyrimidine biosynthetic pathway which supplies the precursors for DNA synthesis in rapidly dividing tumor cells. On the other hand, RT is a unique retroviral DNA polymerase required for the integration of the retroviral genome into host cells. The initial research for this thesis probed the anionic binding site of ATCase for the purpose of further development of bisubstrate/transition state analogs. Several charged tetrahedral analogs that were predicted to exhibit a high affinity for ATCase were synthesized but found to be relatively weak inhibitors, research efforts were then diverted to a second target enzyme. During the course of the ATCase research, the inhibitory effects of small anionic ligands which had previously been tested for inhibition of RT were determined. It was striking that these two diverse enzymes exhibited a similar profile of inhibition by these ligands (Table 1). Three of these ligands, namely phosphonofluoridate (PFA), hypophosphonate and carboxylidiphosphonate appeared to have the properties required for effective inhibition of both enzymes. This may be due to the fact that both enzymes have substrates with phosphate groups and that their postulated transition states involve the development of an additional negative charge. Since PFA was a relatively potent and clinically useful inhibitor of RT but its mode of inhibition of this enzyme was not fully understood, the focus of the research then shifted to RT. A more detailed treatment of the studies conducted on RT and
TABLE II: COMMON INHIBITORS OF HIV-RT AND ATCase

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<th>Compound</th>
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<th>ATCase</th>
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<tr>
<td>R = -PO₄⁻</td>
<td>IC₅₀ (μM)</td>
<td>Ki (nM)</td>
</tr>
<tr>
<td>R=CO₂⁻</td>
<td>0.00035⁺</td>
<td>0.022⁺</td>
</tr>
<tr>
<td>R=CH₂CO₂⁻</td>
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<td>17.5⁺</td>
</tr>
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<td>0.026⁺</td>
</tr>
<tr>
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<td>0.051⁺</td>
</tr>
<tr>
<td>R=OSO₂PO₂⁻³</td>
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</tr>
<tr>
<td>R=CH₂PO₂⁻³</td>
<td>2.6⁺</td>
<td>0.037⁺</td>
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³ Porter et al., 1969. ⁴ Leing et al., 1990.

2. REVERSE TRANSCRIPTASE

2.1 INTRODUCTION

The aim of this research is to probe the active site of reverse transcriptase to further understand the catalytic mechanism and develop potent inhibitors of this enzyme. It is therefore important to review the current knowledge concerning this enzyme.

2.1.1 THE VIRAL LIFE CYCLE

RT is an enzyme present in retroviruses which is coded for by the viral genome. It is quite distinct from other polymerases in that it reverses the flow of genetic information from RNA back to DNA (Crick, 1970; Baltimore, 1970). It catalyzes the synthesis of double stranded DNA from the viral single stranded RNA template by a process that involves three different enzymatic activities: synthesis of the first DNA strand, degradation of the viral RNA in the DNA-RNA hybrid, and synthesis of the second DNA strand. The RNA- and DNA-dependant polymerase activities are apparently performed by the same catalytic moiety, whereas the RNase H activity is mediated by a separate part of the protein (Johnson et al., 1986).

The process of reverse transcription occurs in the early phase of the viral life cycle after virus-cell fusion and release of the virion core into the cytoplasm (Varsmas & Swanstrom, 1984). RT and the viral integrase enzymes remain associated with genomic viral RNA in the form of a nucleoprotein complex. Reverse transcription occurs within this complex in the cytoplasm of the infected cell. The final product is the proviral DNA which is then transported into the nucleus and integrated into the host by the integrase enzyme. Full length linear viral DNA molecules appear in the nucleus about 6-8 hours after infection (Varsmas & Swanstrom, 1984). The late phase starts with the synthesis of viral transcripts from the provirus. Spliced and full-length viral RNA molecules are transported to the cytoplasm, where some are translated to yield viral proteins and polyproteins. Viral genomic RNA then associates with these proteins during the assembly of the core particles. This is followed by budding and release of mature infectious virions.

Reverse transcriptase catalyzes an obligate step in the retroviral life cycle. RT appears to have the same basic requirements and mechanism for chain extension as the cellular DNA-dependent DNA polymerases; they all require a free 3'-hydroxyl group of a primer, and a complementary template; the overall direction of chain growth is 5'→3'; and they require all four dNTP's for DNA synthesis.
(Rohrbach, 1974). However, RTs differ from the DNA polymerases of eukaryotic cells in that they can utilize ribopolymers efficiently and the endogenous reaction of RT is primered by tRNAs. Since no eukaryotic cellular RTs have been identified, RT is an important target for the development of antiviral agents.

2.1.2 GENERAL PROPERTIES OF REVERSE TRANSCRIPTASES

The RTs isolated from a variety of avian and mammalian retroviruses have differences in their structural and catalytic properties. The enzyme from Maclomey murine leukemia virus (MLV) is an 80 kDa monomer. The polymerases and RNase H domains reside on the amino- and carboxy-terminal regions of the enzyme, respectively (Kotewicz et al., 1988). MLV-RT exhibits significantly greater polymerase activity in the presence of Mg+ ions than Mn+ ions, and the enzyme binds several tRNAs with similar affinities.

The RTs from AMV and HIVI differ from MLV-RT in most of the above characteristics. They are both heterodimers with the smaller subunit being generated from a carboxy-terminal cleavage of the larger one (Moelling et al., 1971; Hansen et al., 1988). Both of the subunits of AMV-RT (95- and 63-kDa) have polymerase and RNase H activities (Varaus & Swanson, 1984). In contrast, proteolytic cleavage of the 66 kDa subunit of HIVI-RT (p66) to form the 51 kDa subunit removes polymerization by an ordered mechanism with the template-primer binding first. Synthesis is processive in that multiple dNTP additions can occur before the enzyme terminates by dissociating from the template-primer (Majumdar et al., 1988). The chance of termination is greatest after incorporation of the first dNTP residue, while the probability of termination is lower and stays constant after the third incorporation (Majumdar et al., 1988). This is consistent with the possibility that the initiation of synthesis is kinetically distinct from the elongation steps.

Bacterial and mammalian DNA polymerases also appear to follow a similar ordered mechanism and are also processive with the exception of mammalian DNA polymerase α (Majumdar et al., 1988).

2.1.5 INTERACTION OF POLYMERASE AND RNASE H ACTIVITIES

It is well known that the polymerase and RNase H functions of RTs reside on different domains of the same polypeptide and that they share the template-primer binding site. However, the level of functional interrelation between the two activities has not been well characterized. Mutagenesis and limited proteolysis studies have produced proteins with only one of these catalytic functions (Hansen et al., 1988; Kotewicz et al., 1988). However, certain point mutations in the polymerase domain abolish RNase H activity most of the RNase H domain. Both enzymes generally prefer Mg+ to Mn+ as a divalent cation for substrate activation, and each one preferentially interacts with one tRNA. AMV-RT specifically recognizes tRNA+ while tRNA+ is thought to prime DNA synthesis in HIV infection and has been shown to induce significant conformational changes upon binding to HIVI-RT (Robert et al., 1990).

2.1.3 THE BINDING OF tRNAs TO RT

The specific recognition of tRNAs by the dimeric RTs and not the monomeric RT suggests that the second subunit of the dimer promotes specific binding of the cognate tRNA. This is supported by several studies on HIVI-RT. The crystal structure of the p66/p51 heterodimer has one large cleft for binding the template-primer which is made up primarily by p66 (Kohlstaedt et al., 1992). When an A-form RNA-DNA hybrid is modeled into the large cleft of the heterodimer, the location of p51 implies it could play a role in tRNA binding. In this structure, a specific domain of the p51 which is not conserved amongst other RTs (Poch et al., 1989) would be able to contact the tRNA after the 18 3' nucleotides unpair and base pair with the template.

2.1.4 ORDERED MECHANISM

Kinetic studies on HIVI-RT show that RT catalyzes of HIVI-RT, while mutations in the RNase H domain sometimes affect polymerase activity (Hsi et al., 1989; Prasad & Coff, 1989). When an RNA-DNA hybrid is bound to the active site of HIVI-RT, RNA hydrolysis primarily occurs 15-16 nucleotides away from the site of polymerization in HIVI-RT (Parunio & Beardon, 1991). Studies done on HIVI+, AMV+, and MLV-RT indicate that processive degradation of RNA does not occur during DNA synthesis for all three enzymes, but a limited number of cleavages do occur during DNA synthesis (Bastefano et al., 1991). Those results suggest that the RNase H function is much less active than the polymerisation function during processive DNA synthesis and that the activities are not strictly coupled.

2.1.6 STRUCTURE-FUNCTION RELATIONSHIPS IN HIVI-RT

The best characterized RT is that from HIVI, as the crystal structure has recently been determined and several site-directed mutagenesis studies have been performed on it. The overall structure of HIVI-RT is first discussed followed by a review of the specific subdomains and their presumed function.

The p66 subunit of HIVI-RT is folded into 5 separate subdomains, the R-H domain and four subdomains of the polymerase (Kohlstaedt et al., 1992; Figure 2). The polymerase domains form a structure that resembles a right
hand with the "palm" subdomain forming the base of the protein and the "fingers" subdomain opposing the "thumb" subdomain. The fourth domain is the "connection", so called because it lies between the rest of the polymerase and the RNase H domains. This structure has a large cleft that runs between the RNase H and polymerase active sites and is similar to the cleft in the crystal structure of the Klonev fragment of Pol I (Ollis et al, 1985). The palm domain forms the bottom surface of this cleft. A model-built A-form RNA-DNA hybrid fits into this cleft and can be positioned such that the 3'-terminus is adjacent to the residues thought to be directly involved in polymerization (see below).

The p51 subunit has a strikingly different structure than p66 with no large cleft and the catalytically essential residues (see below) are buried (Kolstaedt et al., 1992). When combined with p66, it forms a dimer with a large degree of asymmetry. Significant conformational changes, as determined by circular dichroism studies, occur in both p66 and p51 when the dimer is formed (Anderson & Coleman, 1992). Kinetic studies on the p66 monomer and on the heterodimer are consistent with a model in which one of the subunit active centers is inactivated upon formation of the heterodimer (Anderson & Coleman, 1992). The Kcat of the heterodimer is one half that of p66, while the Kcat of the polymerase for the template-primer is one fifth of the value for p66. The

resulting twofold increase in the Kcat/Km gives a distinct catalytic advantage to the heterodimer, since the in vivo reaction catalyzed by HIV-1 RT is most likely at rate-limiting substrate concentrations.

2.1.7 CONSERVED REGIONS: THE "PALS" SUBDOMAIN

Initial sequence analysis studies on RTs and RNA polymerases identified a "polymerase signature sequence" within a 14-residue conserved region in these enzymes (Argos, 1988). This motif is characterized by an aspartate doublet flanked by several hydrophobic residues on each side. The conserved aspartate residues are on an exposed loop in a predicted β-hairpin structure (Argos, 1988) suggesting that they somehow participate in catalysis and are not conserved for purely structural reasons. Further sequence analysis of RNA-dependent polymerases identified other conserved regions, including a third invariant aspartate residue within the RTs and RNA polymerases (Argos, 1988).

Mutation analysis of each of the three conserved aspartate residues of HIV-1 RT produced inactive enzymes which were still able to bind template-primer (Tarder et al., 1992; Lowe et al., 1991). Similarly, mutation of the three acidic residues in Pol I reduced polymerase activity to barely detectable levels (Polesky et al., 1990; Polesky et al., 1992). Although Pol I and HIV-1 RT show little overall sequence homology (Delarue et al., 1990), the crystal structures of the Klonev fragment of Pol I and p66 of HIV-1 RT indicated a similar tertiary structure (Ollis et al., 1985; Kolstaedt et al., 1992). The conserved acidic residues in both enzymes are found in a similar orientation in the subdomain which forms the bottom surface of the DNA binding cleft of both enzymes, strongly suggesting that this motif plays a similar key role in nucleotide polymerization in both enzymes. In the E. coli enzyme, the acidic residues have been observed to bind divalent metal ions (Reese & Stitz, unpublished), suggesting that part of the function of this structure is to co-ordinate an essential metal ion(s). The role of metals in RT activity will be discussed in a later section.

2.1.8 THE "THUMB" AND "FINGERS" SUBDOMAINS

The "thumb" and "fingers" subdomains of HIV-1 RT form opposing sides of the DNA binding cleft around the polymerase active site (Kolstaedt et al., 1992; Figure 2). The "thumb" subdomain contains a region (residues 257-266) conserved in RTs (Jancobo-Molina & Arnold, 1991). A lysine residue (K263) within this region has been labeled by an MTV substrate-binding site directed reagent for DNA polymerases (Basu et al., 1989), suggesting it may have a role in binding the substrate NTPs. This basic residue could play a role in opposing the negatively charged oxygens of the triphosphate
society of the substrate. However replacement of lysine 263 with a valine residue did not change the level of inhibition by the triphosphate form of both AT and 2',3'-dideoxyxuanosine (devreese et al, 1992). The fact that a point mutation at position 264 produced an enzyme with a high level of resistance to the nucleoside analog AZT (larder et al, 1987) further implicates a substrate binding function for this region.

The "finger" subdomain of HIV-1 RT is unlike the corresponding subdomain of Pol I in both structure and amino acid sequence (Kohler et al, 1992). The region appears to contact the template strand when a DNA-RNA hybrid is modelled into the active site of the enzyme. Within this region is an invariant glycine residue found in all RNA-dependent polymerases (Poch et al, 1989). Accordingly, this residue may play a role in recognition of the RNA template.

In summary, the recent crystal structure determination of HIV-1 RT in combination with several other types of studies on RTs have shed some light into the mode of reverse transcription and the structure of the active polymerization complex, which should help to characterize the catalytic mechanism of RTs and in the development of specific and potent inhibitors of this viral enzyme.

2.1.9 METAL IONS IN NUCLEOTIDYL POLYMERIZATION

TABLE II

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>SOURCE</th>
<th>ZINC CONTENT (atom/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcriptase</td>
<td>AMV</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Rauscher murine leukemia virus</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Woolly monkey Type C virus</td>
<td></td>
</tr>
<tr>
<td>DNA Polymerases</td>
<td>E. coli</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Sea urchin</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>T4 phage</td>
<td>1.0</td>
</tr>
<tr>
<td>RNA Polymerases</td>
<td>E. coli</td>
<td>2.0</td>
</tr>
<tr>
<td>Deoxyribonucleotidyl</td>
<td>calf thymus</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Early studies on DNA polymerases and RTs reported that metal chelators were effective inhibitors of these enzymes even in the presence of a large excess of Mg" or Mn" ions used to activate the TP substrates (Chang & Bollum, 1970; Slater et al, 1971; Auld et al, 1975). These reports found specific inhibition of the polymerases by ortho-phenanthroline (OP) and a lack of inhibition by its non-chelating isomer meta-phenanthroline. Since OP has a high affinity for Zn", a moderate affinity for Mn" and a low affinity for Mg", this led to the conclusion that DNA polymerases were zinc metalloenzymes. The presence of stoichiometric amounts of zinc was demonstrated not only in DNA polymerases and RTs but also in RNA polymerases (Table II). For both Pol I and AMV-RT, dialysis of the enzyme against OP resulted in a loss of zinc and a parallel loss in activity (Springgate et al, 1973; Poiesz et al, 1974). Activity of both enzymes was restored by the addition of Zn" ions. Similarly, it was found that the metal-free 8 and 8' subunits of E. coli RNA polymerase can be reconstituted with Zn" ions (Wu et al, 1977). These results implied the essentiality of zinc in the action of nucleotidyl polymerases.

A reinvestigation into the metal content of Pol I isolated from either lambda pol A phage-lysogenized E. coli (Walton et al, 1982) or wild type E. coli (Ferrin et al, 1983) found ca. 0.2 g atom zinc/mole of enzyme and that activity did not correlate with zinc content. However, Mg" was present in both enzymes at a level of 0.7 g atom/mole of enzyme (Ferrin et al, 1983), suggesting that either zinc or magnesium ions were required for activity.

2.1.10 OP: AN ALTERNATIVE MECHANISM OF INHIBITION

The mechanism of inhibition of Pol I by OP was reinvestigated and results suggested that it was not due to the complexation of an active site metal, but the formation of an OP-Cu" complex. This complex was reported to catalyze a rapid scission of the template and primer strands, the products of which are potent inhibitors of Pol I (Sigman et al, 1979). It was suggested that trace levels of contaminating Cu" were reduced to Cu" by thiols in the polymerase assay mixture. This was based on the thiol dependency of OP inhibition observed by these authors, and the reversal of this inhibition by the Cu" specific chelator 2,9-dimethyl-1,10-phenanthroline. While this phenomenon may complicate the interpretation of the OP inhibition of Pol I, it does not explain the loss of activity of Pol I which occurs upon dialysis against OP in the absence of template-primer, since activity was measured in a metal-free polymerase assay after removal of OP by dialysis (Springgate et al, 1972).
This alternative mechanism of OP-mediated inhibition cannot account for the inhibition of ANV-NRT by other chelating agents whose metal complexes are unlikely to cleave the template into inhibitory fragments (Auld et al., 1974), nor for the loss of polymerase activity which occurs upon dialysis of this enzyme against OP (Poliesz et al., 1974). In addition, it was recently reported that OP inhibits HIV-1-NRT in the absence of any added thiol (Hizi et al., 1991). Studies on RNA polymerase from wheat germ also concluded that OP inhibits this enzyme in a metal-free assay mixture without added thiol and is therefore due to chelation of a functional zinc ion (Marus & False, 1994). Nonetheless, studies on the interaction of OP with polymerases should be designed so as to avoid the possibility of template cleavage by a Cu^2+ (OP)_2 complex.

The RNase H activity of NRTs have differing susceptibilities to the effects of the metal chelator OP. For the dimeric NRTs from ANV and HIV1, the RNase H activities are relatively resistant to the inhibitory effects of OP, with IC_{50} values at least tenfold larger than the corresponding values for polymerization (Modak & Sreeravastava, 1979; Hizi et al., 1991). This is in agreement with crystal structure studies on the RNase H domain of HIV-1 RT, where two manganese ions are co-ordinated at the active site (Davies et al., 1991). In contrast, MLV-NRT exhibits a distinct inhibition profile with OP. The IC_{50} value for the inhibition of RNase H activity is 50 nM, fivefold lower than that for the polymerase function (Hizi et al., 1991). This suggests that the metal ion (presumably zinc) required for RNase H activity is more accessible to chelation by OP than the one in the polymerase active site of MLV-RT.

2.1.11 NRT INHIBITORS

An enormous research effort has been directed towards the development of specific inhibitors of NRTs that have minimal effects on host cell DNA and RNA polymerases, or other host cell processes. A large variety of compounds have been found to exhibit various levels of potency and specificity for inhibition of NRTs. These inhibitors can be divided into three main categories according to their structure and presumed mode of interaction with NRTs: a) pyrophosphate analogs, b) nucleoside analogs c) non-nucleoside analogs.

2.1.11(a) PYROPHOSPHONATE

Phosphonate is a pyrophosphate analog with demonstrated anti-HIV activity (Sandstrom et al., 1985). Despite its structural and ionic similarity to the triphosphate portion of the substrate NTPs, it has been reported that PFA exhibits non-competitive inhibition of several NRTs with respect to this substrate, suggesting that it binds to a site distinct from the NTP binding site (Erickson et al., 1982; Yang & Cheng, 1986). In addition, studies with certain non-nucleoside inhibitors, which do not bind to the substrate binding site, indicate that PFA may interact with a similar target site as these hydrophobic compounds (see section 2.1.11(c)).

However, it was also reported that PFA inhibits HIV-1-NRT in a mutually exclusive manner with NTP analogs in combined inhibitor studies (Starnes & Cheng, 1988). This would suggest overlapping binding sites for PFA and the substrate NTPs. To add to the controversy, it was found that some but not all of the mutations which decrease the affinity of HIV1-RT for various NTP analogs also decrease PFA binding (Prasad et al., 1991; Lowe et al., 1991). These observations suggest that the site of interaction of PFA overlaps with that of the substrate. Because of these conflicting reports on the mode of binding of PFA, this issue is addressed in the work reported here.

2.1.11(b) NUCLEOSIDE ANALOGS

A family of 2'-3'-dideoxynucleoside analogs are inhibitors of HIV replication within host cells both in vitro and in vivo (Nittaya et al. 1985; Yarchoan et al., 1989). The prototype of this family is 3'-azido-2',3'-dideoxynucleosides (AZT), where the 3'-OH of thymidine is replaced by an azide group. After sequential phosphorylation by the host cell enzymes thymidine kinase, thymidylate kinase and NDP kinase, the triphosphate form of AZT (AZTTP) is a substrate for RT, and AZT-monophosphate becomes incorporated into the growing viral DNA strand. However it causes chain termination because it has no 3'-OH for further esterification. The AZT-terminated template-primer is a potent competitive inhibitor of HIV-1 RT with a K_i of 2.4 nM (Beidenreich et al., 1990).

There are significant limitations to the use of AZT as an antiviral agent for AIDS, including toxic side effects and the emergence of resistant HIV strains within 6-18 months of therapy (Larder et al., 1987). Other dideoxynucleosides are also effective antiretroviral agents, but they also have significant side effects and resistance also develops to these compounds (Matthews et al., 1991). The problems associated with the dideoxynucleosides have given impetus to the development of other types of NRT inhibitors, such as the acyclic nucleotide phosphonates (Figure 3, PMEA and PMPA).

The presence of the phosphonate group eliminates the need for the first phosphorylation step which is the rate-limiting step in the activation process of the dideoxynucleosides. After conversion to their diphasphate forms, these compounds are effective inhibitors of HIV-1 RT, with IC_{50} < 0.1 nM.
Figure 3: The Structure of Various Potent Inhibitors of HIV-RT

Prototype in each of these classes is shown in Figure 3 [V100, RT-80-587, HA924 and pyridoxine (L-597-439)]. All of these compounds specifically inhibit HIV and not human immunodeficiency virus type 2 or other retroviruses (Hu et al., 1994; Peasels et al., 1994; Balsarini et al., 1992; Goldman et al., 1992). Another common feature of these specific HIV inhibitors is their virtual lack of toxicity in cell culture systems and animals. They all exhibit non-competitive inhibition with respect to template, primer or nucleotide.

The observation that pyridoxines are also displaced from the enzyme-template-primer complex by PFA and that they inhibit in a mutually exclusive manner with respect to PFA (Goldman et al., 1991) suggested that they may act at the pyrophosphate binding site or that the binding of pyridoxines kinetically overlaps with that of PFA. However, the mutations associated with pyridoxine resistance generally cause an increased sensitivity to inhibition by PFA (Sardana et al., 1992). It is of importance to elucidate the functional association between the enzyme’s binding sites for PFA and the non-nucleoside inhibitors, and to assess the feasibility of development of an inhibitor taking advantage of the binding energy available to both types of compounds.

2.2 DESIGN OF RESEARCH

The main goal of this research is to probe the active site of different reverse transcriptases with various methods to a) further characterize the binding of anionic compounds to the active site and b) investigate the essentiality and role of a metal in the polymerization reaction. The specific objectives of the research are described below.

2.2.1 ANIONIC INHIBITORS

Previous studies on the inhibition of both HIV-RT and HIV-1 RT by various small anionic ligands have been done. Since PFA was the most effective inhibitor of a variety of compounds tested on both dimeric RTs, it was of interest to determine the susceptibility of a distinct retroviral enzyme (MLV-RT) and a DNA polymerase (Pol I) to this inhibitor. Therefore an extended range of small anionic inhibitors were tested on the three RTs and Pol I in an attempt to further determine the structural basis for the tight binding of PFA to these enzymes. Specifically, the importance of the phosphate and carboxylate moieties of PFA was determined by observing the effect of replacing these groups with others on the ability to inhibit the various enzymes. Since PFA is a highly charged compound, the importance of the negative charges in promoting interaction with the different polymerases was investigated with various cationized derivatives of PFA. It has been suggested that the
inhibitory power of a variety of pyrophosphate analogs against viral polymerases is related to their ability to chelate an active site zinc ion (Glass and Hutchinson, 1980), several inhibitors that incorporate an effective zinc ligand (a thiol or hydroxamate group) were included.

2.2.2 NUCLEOSIDE ANALOGS

The second objective was to investigate the NTP binding site of RTs, particularly the triphosphate binding region. The relative contribution of each phosphate group of the substrate to the binding of the substrate NTPs was studied. A variety of thymidine analogs with both anionic and hydrophobic 5' substituents were tested for inhibition to observe what variations in structure could be accommodated by the RTs. The viral enzymes are known to have less stringent structural requirements for substrate binding compared to cellular DNA polymerases and therefore misincorporation of nucleotides occurs much more frequently with the RTs. Defining the types of 5' substituents that can be tolerated by the RTs should help in the design of an NTP analog that does not require host cell phosphorylation to display anti-HIV1 activity and specifically inhibits RT.

2.2.3 COMBINED INHIBITOR STUDIES

In order to further investigate the mode of binding of PFA and other anionic compounds to HIV1-RT, the concurrent effects of various pairs of inhibitors were investigated. PFA is presumed to interact with RT at the same site as the product pyrophosphate, but this has not been proven. For this reason, the interaction between the product pyrophosphate and PFA within the active site of RT was examined with multiple inhibitor studies. The combined effects of PFA and thymidine, or its mono- and di-phosphate derivatives were measured to look at the relative contribution of each of the phosphate groups to the mutually exclusive inhibition reported for dATTP and PFA (Starnes & Cheng, 1988). Through these studies, the spatial relationship between the PFA binding site and that of the product pyrophosphate or the substrate NTP and its analogs was explored.

2.2.4 OP STUDIES

The last major objective was to perform a more detailed characterization of the inhibitory effect of the metal chelator OP on RTs. It is not known what precise role, if any, zinc or another divalent metal plays in the polymerization reaction by HIV1-RT. Elucidation of this role is of interest because it could aid in the development of potent inhibitors that incorporate a strategically placed metal ligand into their structure. Studies were designed to determine the mode of inhibition of HIV1-RT and MLV-RT by this chelator with respect to both the template-primer and the substrate NTP. The possibility that OP could cause a time-dependent inactivation of HIV1-RT was examined, as was the ability of various metal ions to restore activity to the inactivated enzyme.

Due to the controversy surrounding the mode of inhibition of Pol I and other polymerases by direct exposure to OP, the possibility that the observed OP-mediated inhibition of HIV1-RT was actually due to the effects of an (OP)₂·CN complex was explored. Finally, the mode of inactivation of HIV1-RT and MLV-RT by OP was investigated. The protective effect of various known active site ligands including the small anionic compounds mentioned above on OP-mediated inactivation was determined to aid in the localization of the site of chelation.

In summary, it is hoped that the results from investigating the objectives described above for RT will aid to the body of knowledge concerning the binding functions of this important viral enzyme and aid in the development of specific and potent inhibitors of this enzyme.

2.3 MATERIALS AND METHODS

2.3.1 MATERIALS

[³²P]dATP was purchased from Moravek Biochemicals and was diluted to a specific activity of 2500cpm/pmol with unlabeled dATP. Unlabeled dGTP, poly(dA)·oligo(dT)·, AV-RT and Pol I were obtained from Pharmacia. Purified recombinant HIV1-RT was purchased from Worthington Biochemical Corp. and recombinant MLV-RT was obtained from BRL. Ready-safe scintillation fluid was from Beckman. Dichloroacetethylphosphonate, carbonyl dichlorophosphate, and peroxo-diphosphonate were gifts from Dr. D.W. Hutchinson (U. of Warwick, England). Hydroxymethylchloroacetethylphosphonate, and hypophosphonate were gifts from Dr. B. Öberg (Medivir, Sweden). Anthine was purchased from Fisher and was distilled from zinc dust prior to use. Ethyldithiobenzophenylformate was purchased from Aldrich and was purified before use by diluting 0.5 ml tenfold into distilled water and passing through a column of Dowex-1 (H form). The column was eluted with water, the eluent was adjusted to pH 7 with dilute ammonium hydroxide and then reneutralized to remove the water. Bovine serum albumin, cobalt sulphate, cupric chloride,
2.3.2 ENTIRE STUDIES

2.3.2.1 ENTIRE ASSAYS

HIV-1-RT RNA-dependent DNA polymerase assays were carried out with 0.020-0.040 µL of HIV-1-RT in a final volume of 50 µL at 37°C and had final concentrations of 50 mM Hapes pH 7.8, 5 mM MgCl₂, 50 mM KCl, 2 mM dithiothreitol (DTT), 0.1 mM BSA; 5 µg/ml poly(rA·oligo dT) and 5 µM [³²P]dCTP (Basu et al., 1989). The reaction was initiated by the addition of TTP unless otherwise indicated. After a 30 min incubation period, a 40 µL aliquot was spotted onto a Whatman GFF glass fiber filter (2.5 cm) which was dropped immediately into a flask of cold 10% trichloroacetic acid (TCA) containing 10 mM pyrophosphate. The filters were washed 3 x 5 min in the cold TCA solution (10 ml/filter/wash) and then rinsed 2 x 2 min with absolute ethanol and 1 x 2 min with ether. The dried filters were then placed in scintillation vials with 7 ml of scintillation fluid and counted in a Beckman scintillation counter. HIV-1-RT dilution buffer had the following components: 0.1 mM Hapes pH 7.8, 408 µg/ml, 5 mM MgCl₂ and 20 mM NaCl.

ANV-RT RNA-dependent DNA polymerase assays were carried out in a final volume of 50 µL at 37°C and had final concentrations of 50 mM Tris-Cl pH 8.3, 7.5 mM MgCl₂, 10 mM DTT, 40 mM KCl, 0.1 mg/ml bovine serum albumin, 20 µM [³²P]dCTP and 50 ng/ml of template-primer A,3(3H)T₄.

2.3.2.2 DATA ANALYSIS.

The IC₅₀ values were the average of at least two separate determinations and were obtained by the median effect method (Chou, 1974). Each determination used at least 4 different concentrations of inhibitor, and the results were plotted on a median effect plot (log of activity/ % inhibition vs log inhibitor concentration). Regression analysis of this plot allowed the calculation of the IC₅₀ value, where [log activity/ % inhibition] = 0. There was usually agreement between the two IC₅₀ values within 20%.

2.3.2.3 MULTIPLE INHIBITOR STUDIES.

The concurrent effects of two inhibitors were determined by a modified Yonetani and Theorell method (Yonetani & Theorell, 1964), which yielded a quantitative parameter (β) for the interaction between the two compounds. A modified method was used because the normal Yonetani & Theorell plot involved determining enzyme activity in the presence of five different concentrations of one inhibitor in combination with four different concentrations of the second inhibitor. In the effort to minimize consumption of HIV-1-RT enzyme, an alternative to this method was sought. Algebraic manipulation of the basic Michaelis-Menten equation using the concepts of Yonetani & Theorell, it was possible to derive equations which allowed the calculation of β from fewer...
assays. The following is the Michaelis-Menton equation:

\[ V = \frac{V_{\max} [S]}{K_m + [S]} \]

Since a competitive inhibitor will affect the apparent \( K_m \) of the substrate by a factor of \((1 + 1/K_i)\), and two competitive inhibitors (1 and 2) will affect the apparent \( K_m \) by a factor of:

\[ \frac{1 + \frac{1}{K_{i1}} + \frac{1}{K_{i2}}}{K_{i2} + \frac{1}{K_{i1}} + \frac{1}{K_{i2}}} \]

the velocity of an enzyme reaction in the presence of one or two inhibitors can then be calculated using the Michaelis-Menton equation and the appropriate factor. Algebraic manipulation of the resulting equations for velocity in the presence of no inhibitor, two inhibitors and each of the two inhibitors separately yielded the following equation:

\[ \Delta K_m = \frac{[I] / (1/K_m - 1/K_{i2}) - (1/K_m - 1/K_{i1})}{1/(1/K_m - 1/K_{i1}) - (1/K_m - 1/K_{i2})} \]

In each experiment two different concentrations of each inhibitor were used separately and in combinations, and the results yielded 4 estimates for the \( \Delta K_m \) value using the above equation. The reported values are the average of at least two separate experiments for each combination of inhibitors.

2.3.2.4 TIME-DEPENDENT INACTIVATION OF HIV-1-RT.

HIV-1-RT was diluted into HIV-1-RT buffer and incubated ± OP (0.5 mM) at 0°C or 37°C for 30 min. At 10 min intervals aliquots were removed and diluted ten-fold into an activity assay. Samples which were preincubated without OP had 50 µM OP added to the activity assay to keep the background levels of OP constant for all samples.

2.3.2(e) PROTECTION EXPERIMENTS.

For the protection studies, aliquots of HIV-1-RT or HIV-RT were preincubated in dilution buffer ± template-primer (50 µg/ml) and ± second ligand for 10 min at 37°C. The ligand concentrations were at least twice the \( K_m \) or IC50 values and were 20 mM TTP, 2 mM PFA and 0.5 mM PPF for HIV-1-RT and 20 mM TTP, 10 mM PFA and 0.70 mM PPF for HIV-RT. The inactivation was initiated by the addition of OP (1.0 mM for HIV-1-RT, and 0.5 mM for HIV-RT) and after 60 min at 37°C was stopped by diluting an aliquot 20-fold into activity assays. OP was omitted from the control reactions.

2.3.2(f) REACTIVATION STUDIES.

Each enzyme was incubated in dilution buffer and OP for 60 min at 37°C and then diluted ten-fold into buffer ± metal for 2 h at 0°C. An aliquot was then removed and diluted five-fold into polymerase activity assays. Full activity (100%) was obtained by incubating each enzyme without OP and diluting into metal-free buffer. The OP concentrations during inactivation were 1.0 mM for HIV1-RT and 0.5 mM for both MLV-RT and Pol I. The metal concentrations during the reactivation studies were 0.11 mM for HIV1-RT and 0.056 mM for both MLV-RT and Pol I. The MLV-RT polymerase activity assay for the reactivation studies contained 2 mM Mg2+ in place of the substrate activating Mg2+ ion which is normally used for this enzyme (Verma, 1979). This was necessary in order to study the ability of various metals including Mg2+ to reactivate the polymerase function of the OP-inactivated enzyme.

2.3.2(g) INHIBITION OF Cu2+ AND Zn2+-ACTIVATED HIV1-RT.

HIV1-RT was inactivated by incubating in HIV1-RT buffer with OP (1.5 mM) for 60 min at 37°C. The enzyme was then diluted tenfold into buffer containing 0.16 mM of Cu2+ or Zn2+ and stored on ice for 2 hours. The enzyme was then diluted tenfold into a standard HIV1-RT activity assay containing the inhibitor, and the activity was measured as described above.

2.3.3 INHIBITOR SYNTHESIS

2.3.3(a) ANALYTICAL CHEMISTRY.

Melt points were determined on a Fisher-Johns hot-stage apparatus and were uncorrected. The progress of the synthetic reactions were analyzed by thin-layer chromatography (TLC) on silica gel 60, or polyethyleneimine cellulose. Plates were analyzed by exposure to iodine vapour, ferric chloride/HCl spray (for hydroxamates), cysteine spray (for thymidine derivatives, Buchanan, 1951) and ferric chloride/sulphasalicylic acid spray for PPA compounds (Wade & Morgan, 1955).

2.3.3(b) ETHYL OXALATE.

Ethyl oxalyl chloride (1.0 mmol) was converted to sodium ethyl oxalate by adding it to 10 mM HEPES buffer, pH 7.6 (10 µl) and adjusting the pH to 7.6 with 10 mM NaOH.

2.3.3(c) FORMICODIENESTER.

This compound was prepared according to the method of Fishbein et al (1969). Ethyl formate (0.01 mol) was added dropwise to 5 ml of a 2 M hydroxylamine/4 M HCl/25% ethanol solution at 0°C. The mixture was stirred at 22°C for 2 h. After cooling to 0°C, concentrated HCl was added to bring the pH to 6. The mixture was then evaporated to dryness, and then 10 ml of ethanol was added and then the
mixture was again roteoevaporated to dryness. The crystalline residue was extracted twice with 10 ml of boiling ethyl acetate. After the addition of acetone (0.5 ml) the extract was reduced in volume to yield white crystals (913 yield), mp 77-79°C (lit. mp 76-79°C; Fishbein et al., 1969) and δ 7.74 ppm in 25 FeCl3/0.1 N HCl (lit. δ 0.772 ppm; Fishbein et al., 1969).

2.3.3(d) 5′-O-METHYLENE-DIPROPHENYL-8-D-2-(3,5-DINITROPHENYL)-THYMINE

This compound was prepared according to the method of Stock (1979). Methylenediphosphonic acid (1.2 mmol) was dissolved in a mixture of DMF (6 ml) and triethylamine (4.8 mmol). 5′-Tocylthymidine (0.6 mmol) was added and the mixture was heated for 6 h at 100°C. The solvent was removed by roteoevaporation at 60°C, and then the mixture was passed down a column of Dowex-50 (NH4+ form) and eluted with water. The fractions containing thymidine derivatives were combined and taken to dryness on a rotary evaporator. The mixture was chromatographed on silica gel with n-propanol-2 N aqueous ammonia (1:1) as the solvent. Homogenous fractions (as determined by TLC) were combined, evaporated and converted to the sodium salt with Dowex-50 (Na+ form). Product containing fractions were evaporated to a small volume and ethanol was added. The precipitate was crystallized from 95% ethanol. NMR (D2O) δ 1.67 (s, 3H, CH3), 1.81 (t, 2H, -CH2-), 2.07-3.18 (m, 5H, -CH2-).

2.3.3(g) SODIUM (ETHYLCARBONYL)PHOSPHONATE

Selective dealkylation of triethyl phosphonofluorane was performed according to Norita et al (1981). Trimethylsilyli chloride (0.033 ml) was added dropwise to a mixture of triethyl phosphonofluorane (0.026 mol) and potassium iodide (0.033 mol) in dry acetonitrile (2 ml). The flask was capped with a calcium chloride drying tube and stirred for 30 min at 22°C. The potassium chloride was filtered off and washed with dry ether. The filtrate was rotary evaporated to dryness. The oily residue was dissolved in dry methanol (15 ml), titrated with HCl to pH 2.0 and loaded onto a column of Dowex-1 (chloride form). Distilled water (50 ml) was added to wash the column and then a LiCl gradient (0-0.1 M) was applied to the column. Fractions which contained hydroxamate as determined by a ferric chloride test were pooled, titrated to pH 8.0 with LiOH and evaporated to dryness. The LiCl was removed by washing several times with a dry methanol-acetone solution (1:4). Yield was 0.106g or 35%. The visible spectrum of the ferric complex in a 1% FeCl3/1.2 HCl solution gave ε 277 cm⁻¹ at 502 nm. The UV spectrum (1% KOH) yielded an extinction coefficient of 9010 cm⁻¹ m⁻¹ at 218 nm. The preparation used for inhibitor studies was 99% pure by weight according to the values of Anderson et al (1984) for the visible (ferric complex) and UV spectra.

2.3.3(h) SODIUM (ETHYLCARBONYL) PHOSPHONATE

This compound was made with the same method as for the anilinium salt of ethyl phosphonofluorane, using trimethyl phosphonofluorane as the starting ester. The salt was recrystallized twice from absolute ethanol, mp 131-132°C, yield 78%. NMR (D2O) δ 3.6 (s, 3H, CH3), 7.0-7.4 (m, 5H, aniline). The anilinium salt was converted to the sodium salt with Dowex-50 (Na') as described for sodium (ethylicarbonyl)phosphonate. The sodium salt was 99% pure by weight according to the hydroxylamine/FeCl3 assay for esters, and contained <0.5% of PFA according to polyethyleneimine TLC.
2.3.3(1) 5'-O-TOSYL-3-D-2'(DEOXYRIBOFURANOSYL) THYMINE.

A solution of 2.1 mmol of thymidine in 2.5 ml of dry pyridine was cooled to 0°C, and 2.1 mmol of p-tosyl chloride in 2.5 ml of dry pyridine was added dropwise with stirring. The reaction mixture was stored at 0°C for 16 h, and then was decomposed with a small piece of ice and poured into 12 ml of ice water. The resulting white precipitate was collected by suction filtration and thoroughly washed with distilled water. Recrystallization from 95% ethanol gave 1.51 mmol (72% yield) of white crystals, mp 169-170°C (lit. mp 172°C; Nichelson & Todd, 1955); NMR (CDCl$_3$, CD$_3$OD, CD$_3$CN) δ 1.95 (s, 3H, CH$_3$-5), 2.15-2.40 (m, 2H, H-$^5'$), 2.50 (s, 3H, aryl-CH$_3$), 4.00-4.20 (m, 1H, H-$^4'$), 4.2-4.4 (m, 3H, H-$^2'$, H-$^3'$), 6.30 (t, 1H, H-$^1'$), 7.45 (d, 2H, C-1, C-5), 7.85 (d, 2H, C-2, C-4).

<table>
<thead>
<tr>
<th>Structure of inhibitor</th>
<th>Inhibition constant* (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R$_1$-O-C$_5$-R$_2$</td>
<td></td>
</tr>
<tr>
<td>R$_1$ R$_2$</td>
<td>HIV-RT AMV-RT MLV-RT Pol I</td>
</tr>
<tr>
<td>CH CH (PFA) 0.35 (0.5)$^t$</td>
<td>7.5 (8)$^t$ 5.5 71</td>
</tr>
<tr>
<td>OCH$_3$ CH CH</td>
<td>14 50 2500 2600</td>
</tr>
<tr>
<td>OCH$_3$ CH CH</td>
<td>24 (100)$^t$ 66 2500 4000</td>
</tr>
<tr>
<td>OCH$_3$ OCH$_3$ CH</td>
<td>370 (44)$^t$ 5000 &gt;2×10$^4$ &gt;2×10$^4$</td>
</tr>
<tr>
<td>CH$_3$ CH CH</td>
<td>(100)$^t$ ND ND ND</td>
</tr>
<tr>
<td>OCH$_3$ OCH$_3$ CH</td>
<td>(500)$^t$ ND ND ND</td>
</tr>
</tbody>
</table>


2.4 RESULTS

2.4.1 ANIONIC INHIBITORS OF POLYMERASES

2.4.1 (a) INHIBITION BY ESTERIFIED DERIVATIVES OF PHOSPHONIC ACID.

The most distinctive feature of the PFA molecule is its highly charged character. It was therefore of interest to investigate the importance of the negative charges of PFA in promoting interaction with the putative pyrophosphate binding site of RTs. The inhibition of three RTs and Pol I by PFA and its esterified derivatives is presented in Table III.

The results confirm those of previous studies indicating that both HIV-1-RT (Vrang and Øberg, 1986) and AMV-RT (Eriksson et al., 1982) are effectively inhibited by PFA. It is apparent that MLV-RT, a monomeric enzyme, is also sensitive to this inhibitor, with an IC$_{50}$ close to that of the dimeric AMV enzyme. The efficacy of PFA inhibition is therefore not dependent on the subunit structure of RT.

It was determined that Pol I was 10 to 200-fold less sensitive to the inhibitory effects of PFA than the RTs. Therefore, the bacterial DNA polymerase is similar to mammalian DNA polymerases in being less susceptible to the effects of PFA compared to the viral enzymes (Wondrak & Kurth, 1988).

Reversion of only the carboxyl moiety of PFA resulted in a significant loss of potency for all four polymerases, with the least effect on AMV-RT. However, these derivatives are still relatively good inhibitors of both HIV-1-RT and AMV-RT. It is unlikely that the inhibition by these compounds is due to contamination with a small amount of PFA, since the ratio of the IC$_{50}$ values for PFA and its monoaesters is different for each enzyme. For example, the ratio of the IC$_{50}$ values for ethyl phosphonate: PFA ranges from 10 for AMV-RT up to 450 for MLV-RT. If contaminants PFA was responsible for the inhibition observed with the ethyl derivative, the ratios would be the same for each enzyme.

It was interesting that the IC$_{50}$ values for the carboxyl ester derivatives of PFA were similar for the two monomeric enzymes MLV-RT and Pol I, while the two dimeric RTs were much more susceptible to inhibition by these compounds. This suggests that the negatively charged carboxylate moiety may play a greater role in the binding of PFA to the monomeric enzymes. Alternatively, the PFA binding site of the dimeric enzymes is different in that it can accommodate a hydrophobic methyl or ethyl group in the carboxylate region.

Two compounds tested by Vrang and Øberg (1986) with a charge on the phosphonate moiety of PFA eliminated were
included for comparison purposes. The level of inhibition by these compounds suggests that both negative charges on the phosphonate group are also important for strong interaction with HIV-1 RT.

Since it was determined that elimination of one of the charges of PFA reduces its inhibitory capacity, it was expected that the fully esterified derivative would be an even less effective inhibitor. This trend apparently holds for all four enzymes. However, in preliminary experiments with triethylphosphonoformate, the IC₅₀ value for HIV-1 RT was found to be <100 μM, which was surprisingly low for an uncharged pyrophosphate analog. In order to eliminate the possibility that this low value was due to contaminants from hydrolysis of one or more of the outer groups, the triethyl derivative of PFA was treated with ion exchange resin to remove any charged contaminants.

The purified preparation of triethylphosphonoformate was a weaker inhibitor than ethyl phosphonofomate for all four enzymes (Table III). The presence of charged impurities in the fully esterified derivatives is the most likely explanation for the comparatively low IC₅₀ values for the trimethyl and triethyl derivatives of PFA previously obtained for HIV-1 RT (Wondrak and Barth, 1988). By comparing the inhibitory potency of ethyl phosphonofomate to that of triethylphosphonoformate, one can assess the importance of both free oxygens on the phosphonyl group of PFA for binding.

Esterification of the phosphonyl oxygens causes a 75-fold increase in IC₅₀ for AMV-RT but only a 15-fold increase for HIV-1 RT. Thus the free phosphonate group of PFA appears to be somewhat more important for binding to AMV-RT than HIV-1 RT. The fact that both PFA and its triesterified derivative are more effective inhibitors of HIV-1 RT than AMV-RT by 10-20 fold may be because of stronger interaction of the carbonyl and P=O substituents of PFA with the HIV enzyme.

These results underline the importance of the negative charges on both the phosphoryl and carbonyl moieties of PFA in promoting interaction with the RT.

2.4.1 (b) SEPARATION BETWEEN THE TWO ACIDIC GROUPS OF PFA

The results in Table IV illustrate the effects of compounds with increased separation between the acidic groups on the potency of inhibition by PFA. It is known that increasing the separation between the phosphonate and carboxylate groups of PFA decreases inhibition for HIV-1 RT and AMV-RT (Vrang & Øberg, 1986; Eriksson et al., 1989). The same effect is seen, to varying degrees, for MLV-RT and Pol I. The effect is the smallest for the bacterial DNA polymerase (10-fold), while the effect on both AMV-RT and MLV-RT is larger, causing a 70-100 fold loss in inhibitory capacity. However insertion of a methylene group into PFA to form phosphonoacetate causes a dramatic (4000-fold) drop in inhibitor potency for HIV-1 RT. The addition of a second methylene bridge to form phosphonoacetate causes a further drop (5 to 25-fold) in ability to inhibit all polymerases.

This suggests that the distance between the negative charges of PFA is a critical determinant of inhibitor affinity for the RTs and Pol I, but is especially important with the HIV enzyme. However it should be noted that part of the difference in inhibition levels seen with PFA and phosphonoacetate could be due to the different charged states of the two compounds at the pH range of the polymerase assays (7.8 to 8.3). Since the third pK, value for PFA is 7.27 (Warren and Williams, 1971) and the value for phosphonoacetate is 8.6 (Bocey, 1979), PFA is in a more highly charged form than phosphonoacetate in the activity assays. Therefore part of the difference of inhibition seen with these two anionic compounds may be due to differences in the overall number of negative charges available for interaction with the pyrophosphate binding site.

It has been proposed that the ability of a variety of pyrophosphate analogs to inhibit viral DNA and RNA polymerases is related to their ability to form stable chelates with a zinc ion at the active centers of these enzymes (Hutchinson et al., 1986). Since it is known that hydroxamates are effective metal ligands, the synthesis of

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition constant (IC₅₀)</th>
<th>HIV-1 RT</th>
<th>AMV-RT</th>
<th>MLV-RT</th>
<th>Pol I</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-P-O₄⁻</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-CO₂⁻</td>
<td>0.00035 (0.002)</td>
<td>0.0075</td>
<td>0.008</td>
<td>0.0055</td>
<td>0.071</td>
</tr>
<tr>
<td>-CH₃CO₂⁻</td>
<td>1.4 (0.5)</td>
<td>0.50 (22.0)</td>
<td>0.60</td>
<td>0.60</td>
<td>0.90</td>
</tr>
<tr>
<td>-CH₂CH₂CO₂⁻</td>
<td>7 (0.5)</td>
<td>13</td>
<td>10</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>-CH₂CH₂OH</td>
<td>2.0</td>
<td>5.3</td>
<td>2.2</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>-CH₂CH₂NH</td>
<td>5.4</td>
<td>11</td>
<td>8.1</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>-CH₂CH₂CO₂⁻</td>
<td>10</td>
<td>5.7</td>
<td>3.2</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>-CH₂CH₂CN</td>
<td>6.3</td>
<td>17</td>
<td>12</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>-CH₂CH₂CN</td>
<td>9.6</td>
<td>20</td>
<td>6.8</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

phosphonoformhydroxamate and phosphonoacetohydroxamate was attempted. The former compound proved to be very unstable during the purification process and was not isolated. The latter compound (phosphonohydroxamate) was successfully synthesized and tested for inhibition of the polymerases (Table IV). Introduction of the hydroxamate group did not improve inhibitor potency for the RTs, while there was a slight improvement for the inhibition of Pol I. The analog of phosphonacacetate with a -CH₂OH in place of the carbonyl group was also tested for inhibition of the polymerases, as thiols are also effective metal ligands. This compound was a weaker inhibitor (4- to 20-fold) than phosphonoacetate for all enzymes. These results indicate that interaction with an active site metal may not be an important factor in determining the strength of binding of phosphonoformate and its derivatives to RTs.

Three different phosphate esters with structures similar to that of phosphonopropanoate were tested for inhibition of the enzymes. Phosphoglycolic acid was a weak inhibitor of all enzymes. Phosphonol pyruvate and 8-cyanonethyl phosphate were also weak inhibitors of all enzymes, presumably because of their larger size compared to phosphonoacetate.

2.4.1 (c) The Effect of Pyrophosphate Analogs.

PFA is presumed to interact with polymerases at the binding site for pyrophosphate, a product of the polymerase reaction catalyzed by RT. It was therefore of interest to determine the effect of replacing the carboxylic moiety of PFA with a phosphate or various phosphate groups (Table V).

It is well known that pyrophosphate is a much less effective inhibitor than PFA for both AMV-RT and HIV-1-RT (Vrang & Öberg, 1986; Eriksson et al., 1981), and the same trend holds for both MLV-RT and Pol I. It is clear that increasing the distance between the phosphorus atoms in pyrophosphate by the insertion of an extra bridging oxygen causes a significant loss in inhibitor potency for all four enzymes (Table V). On the other hand, elimination of the bridging oxygen had a beneficial effect for all enzymes except MLV-RT. It is unclear why MLV-RT is anomalous in this respect. It is important to note that despite their overall similarity in size, PFA is a better inhibitor than hypophosphonate by a 20-100 fold margin for the RT's and only a 5-fold margin for Pol I. This shows that the preference for a carbonyl group over a phosphate group is more pronounced with the viral enzymes.

Substitution of the bridging oxygen of pyrophosphate with a variety of methylene groups generally caused no change or an increase in nitrogen or the IC₅₀ value for all of the enzymes (Table V). There is, however, a significant drop in IC₅₀ value upon substitution of the bridging oxygen of pyrophosphate with a carbonyl group which is unique to the RTs. This effect is particularly striking for HIV-1-RT. Carbonyl diphosphonates inhibit HIV-1-RT with a potency 75-fold that of pyrophosphate, while the corresponding values for AMV-RT and MLV-RT are 4- and 5-fold respectively. This implies that the carbonyl group promotes additional interactions with the active site of RT or possibly the insertion of the carbonyl moiety changes the orientation of the phosphate groups to allow tighter binding. It is therefore apparent that the carboxylate group of PFA cannot be replaced by a phosphate or phosphonate group, and that the carbonyl group may be important for strong interaction with the RTs.

2.4.1 (d) Inhibition by Carbonyl Compounds.

The effects of carbonyl compounds without a phosphate group on the polymerases were also investigated (Table V). Oxalate, which has a carbonyl group in place of the phosphonate group in PFA, has previously been shown to be a good inhibitor of HIV-1-RT (Vrang & Öberg, 1986) but a much less effective inhibitor of AMV-RT (Eriksson et al., 1981). In our experiments, oxalate acid exhibited a significantly lower IC₅₀ value for HIV-1-RT than that reported by Vrang and Öberg (1986) although this may be due to the different assay conditions used. The polymerase assay used in this research

### Table V

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition constant (IC₅₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV-1-RT</td>
</tr>
<tr>
<td>R -PO₂⁻</td>
<td></td>
</tr>
<tr>
<td>-CO₂⁻ (PFA)</td>
<td>0.00035</td>
</tr>
<tr>
<td></td>
<td>(0.0005)</td>
</tr>
<tr>
<td>-PO₃⁻</td>
<td>0.11 (0.5)</td>
</tr>
<tr>
<td></td>
<td>(0.050)</td>
</tr>
<tr>
<td>-OPO₃⁻</td>
<td>1.5</td>
</tr>
<tr>
<td>-SPO₃⁻</td>
<td>0.08 (&gt;0.5)</td>
</tr>
<tr>
<td>-CH₂PO₃⁻</td>
<td>2.9 (0.5)</td>
</tr>
<tr>
<td>-CCl₃PO₃⁻</td>
<td>2.4 (&gt;5.5)</td>
</tr>
<tr>
<td>OH</td>
<td></td>
</tr>
<tr>
<td>[-CH₂OH]PO₃⁻</td>
<td>3.5 (&gt;5.5)</td>
</tr>
<tr>
<td>[-O(0)PO₃⁻</td>
<td>0.0015 (0.004)</td>
</tr>
</tbody>
</table>

Table VI
Inhibition by small carboxylate derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>HTV-RT</th>
<th>AMV-RT</th>
<th>MLV-RT</th>
<th>Pol I</th>
</tr>
</thead>
<tbody>
<tr>
<td>'OCOOH'</td>
<td>0.011 (0.1)</td>
<td>0.8 (&gt;5)</td>
<td>2.1</td>
<td>4.1</td>
</tr>
<tr>
<td>'OCOOC(OH)CH₃</td>
<td>0.023</td>
<td>0.9</td>
<td>1.9</td>
<td>13</td>
</tr>
<tr>
<td>'OCO(OH)NH₂</td>
<td>0.070</td>
<td>4.2</td>
<td>2.7</td>
<td>10</td>
</tr>
<tr>
<td>'OCOONa</td>
<td>5.0</td>
<td>5.0</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>'OCOH₂</td>
<td>95</td>
<td>21</td>
<td>71</td>
<td>85</td>
</tr>
<tr>
<td>'OCOONH₄</td>
<td>19</td>
<td>6.1</td>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td>H(O)CNH₂OH</td>
<td>4.1</td>
<td>0.060</td>
<td>1.2</td>
<td>70</td>
</tr>
<tr>
<td>CH₃C(OH)NH₂</td>
<td>70</td>
<td>12</td>
<td>65</td>
<td>66</td>
</tr>
</tbody>
</table>


These results identify two important structural features of oxalic acid, a free carboxylate and an adjacent carbonyl group, which appear to be necessary for effective inhibition of HTV and Pol I.

An unusual finding was that formyloxynoxime inhibited AMV-RT in a strong and selective manner. This effect appears to be bulk-dependant since both acetohydroxamate and oxalyl hydroxamate have 200- and 70-fold larger IC₅₀ values than formyloxynoxime, respectively. It is important to note that this selective inhibition of AMV-RT may be partly due to differences in the fraction of formyloxynoxime molecules which are charged at the pH of each enzyme assay. Since formyloxynoxime has a pH of 8.45 (Fishbein et al, 1969), at the pH of the AMV-RT assay (8.3) there is a larger percentage of molecules which are charged than at the pH of the other enzyme assays (7.6-8.0). If only the charged form of formyloxynoxime were able to inhibit these polymerases, this could be part of the reason why AMV-RT is more sensitive to this compound. It is also possible that the formyloxynoxime ion is binding to a very small cleft unique to AMV-RT.

The strong inhibition of HTV-RT by oxalic acid is apparently not dependent on formation of a stable metal chelate. Thioglycolate is a more effective metal binding agent than oxalic acid (Sillen, 1964) but it is a much weaker inhibitor of HTV-RT (Table VI). A similar but much smaller trend is seen for the other two RTs and Pol I. Therefore there is a specific interaction of the second carbonyl group of oxalic acid with the enzyme that promotes tight binding.

2.4.1.4 COMBINED INHIBITOR STUDIES WITH HTV-RT: PFA.

In order to further investigate the mode of binding of PFA and other anionic compounds with HTV-RT, the concurrent effects of various pairs of inhibitors were investigated. In these experiments, the concentration of both inhibitors was varied and the interaction constant (a) for each pair of ligands was determined. The large interaction constants for the inhibition by PFA in combination with the product pyrophosphate or its analogs carbaryl diprophosphate and oxalate indicate antagonistic binding or a similar mode of action for these compounds (Table VII). This supports the notion that PFA inhibits RT as a pyrophosphate analog (Bunquist & Öberg, 1979).

Phosphonothioate inhibits RT in a non-competitive manner with respect to substrate MTPs (Vrang & Öberg, 1986; Eriksson et al, 1982), suggesting that the binding sites for PFA and MTPs do not overlap in the active site of RT. It should be noted that these experiments used Lineweaver-Burk plots to evaluate the mode of inhibition and the results can be interpreted in an ambiguous manner regarding the point of intersection of the lines and the competitiveness of the
TABLE VII
The Combined Effects of PFA and Other Ligands

<table>
<thead>
<tr>
<th>Ligand 1</th>
<th>Ligand 2</th>
<th>Interaction constant (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFA</td>
<td>pyrophosphate</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>PFA</td>
<td>carbonyl diposphonate</td>
<td>7.2 ± 2.7</td>
</tr>
<tr>
<td>PFA</td>
<td>oxalate</td>
<td>9.5 ± 3.1</td>
</tr>
<tr>
<td>PFA</td>
<td>thymidine</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>PFA</td>
<td>thymidine monophosphate</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>PFA</td>
<td>TTP</td>
<td>44 ± 20</td>
</tr>
<tr>
<td>PFA</td>
<td>2',3'-dideoxy TTP</td>
<td>8</td>
</tr>
</tbody>
</table>

* The interaction constants are reported as the average ± the standard deviation obtained in two separate experiments that each yielded four estimations of the $k$ value.

"Starnes and Cheng (1988)."

Inhibition observed. Site-directed mutagenesis experiments on HIV-1-RT have suggested that PFA interacts with a site very close to the NTP binding region, since some of the mutations that alter the $K_m$ for TTP also affect the $K_m$ for PFA (Lowe et al., 1991). Unlike PFA, the triphosphate forms of the nucleoside analogs 5'-azido-3'-deoxycytidine (AZT) or 2',3'-dideoxycytidine (ddC) are competitive inhibitors with respect to substrate dNTP (Bearden & Miller, 1990) and therefore interact directly with the NTP binding region of RT. However, it was previously reported that PFA inhibits HIV-1-RT in a mutually exclusive manner when combined with either dATTP or dGTP, indicating that the binding of PFA and the nucleoside compounds overlap in a physical or kinetic mode (Starnes & Cheng, 1988). In order to resolve these differences, the inhibition of HIV-1-RT by PFA in combination with various phosphorylated forms of thymidine was evaluated (Table VII).

PFA and thymidine are mutually nonexclusive inhibitors (anti) and can bind simultaneously to the enzyme. The absence of antagonism between these two compounds suggests that a novel thymidine-5'-phosphonofluoromethyl derivative may be an effective inhibitor of HIV-1-RT. This type of compound could take advantage of the binding energy available to the base and ribose moieties of the thymidine molecule and also that for PFA. This type of a compound may also display greater specificity for the inhibition of the viral enzymes when compared with the currently used modified nucleosides. Modified nucleosides with a 5'- substituent of PFA or phosphonocarboxylate have been synthesized and tested for activity against herpes simplex virus (Lambert et al., 1989), since these pyrophosphate analogs are effective inhibitors of the polymerase induced by this virus. The attachment of PFA to the 5'-position of the antiviral agent 5-bromo-2'-deoxyuridine formed a compound with slightly greater antiviral activity than either compound separately (Lambert et al., 1979). Therefore the synthesis of thymidine-5'-PFA was attempted by the coupling method of Lambert (Lambert et al., 1989). During the initial stages of the synthesis, a group from Harvard published a report on the successful synthesis of the 5'-PFA derivatives of AZT and 2',3'-dideoxyuridine for the purpose of testing their anti-HIV activities (Saha et al., 1991). The synthesis of the thymidine derivative was therefore continued, although the antiviral activities of these derivatives have not yet been published by this group.

There is a limited antagonism which develops between TDF and PFA (Table VII). This raises the possibility that PFA has more than one mode of binding to HIV-1-RT and that in the presence of TDF the less preferred mode is used, which does not result in the overlapping of the binding sites of TDF and PFA. Alternatively, this incomplete antagonism may be because the binding sites for TDF and PFA are adjacent to each other in the active site of HIV-1-RT. Both compounds are negatively charged, and thus simultaneous binding of both compounds to HIV-1-RT would be significantly but only partially hindered by electrostatic repulsion.

The essentially complete antagonism which develops between TDF and PFA is similar to that found with AZT and PFA (Starnes & Cheng, 1988), and suggests that the binding site for PFA overlaps with the 5' and 3'-phosphate sites of the substrate binding region of HIV-1-RT. It should be noted that these results do not rule out the possibility that PFA binds to a form of the enzyme which does not bind nucleosides with two or three phosphate groups and vice versa, and therefore conformational changes of the enzyme prevent simultaneous inhibition by these compounds. Nonetheless, these results support the notion that PFA interacts with the 5' and 3'-phosphate sites of HIV-1-RT and they call into question the interpretation of obervations made by workers regarding the non-competitive nature of inhibition by PFA.

2.4.1 (f) ADENINE THERMINE STUDIES WITH HIV-1-RT: THE VARIATE ION

The adenine ion resembles the phosphate ion in that the three pK values for the ionizations of these compounds are...
similar (Segar, 1975). This anion inhibits a variety of enzymes that catalyze phosphoryl transfer reactions, presumably by virtue of the ease with which it can expand its coordination shell to assume a structure resembling the transition state of these enzymes (Knowles, 1980). Thus ribonucleoside-vanadyl complexes are inhibitors of certain ribonucleases and are thought to be stable analogues of the cyclic 2',3'-monophosphate transition states of these enzymes (Lienhard et al., 1971), although the exact structures of the inhibitory vanadyl complexes have not yet been elucidated. The RNase H activity of RT is presumed to have a different transition state than the above group of ribonucleases, but the RNase H activities of both AMV-RT and HIV-RT are inhibited by vanadyl complexes with Kᵢ values of 2-3 mM (Krug & Berger, 1991). Surprisingly, these complexes also inhibited the polymerase reactions of the two RTs with Kᵢ values of 1-3 mM. It was therefore of interest to characterize the interaction of HIV-RT with the vanadate ion alone and in combination with various nucleosides to determine if it may interact as a transition state analog. This would help to determine the feasibility of developing a deoxynucleoside-vanadate ester that may be a tight binding inhibitor of RT.

The median effect plot (Chou, 1975) for the IC₅₀ determination of the vanadate and phosphate anions is presented in Figure 4. The vanadate ion was a 50-fold better inhibitor of HIV-RT than phosphate, with an IC₅₀ value of 0.28 mM. In addition, the vanadate ion was a more effective inhibitor of HIV-RT polymerase activity than the ribonucleoside-vanadyl complexes (Krug & Berger, 1991). Since the slope of the median effect plot indicates the molecular order of inhibition, and the slope of vanadate is 2, there is more than one binding site for this anion on HIV-RT.

In order to investigate the spatial relationship between the vanadate binding site(s) and that of PFA, combined inhibitor studies were performed (Table VIII). PFA and vanadate have essentially additive effects on HIV-RT and can therefore bind simultaneously to different sites on the enzyme. The observation that vanadate and TMP also bind in a mutually nonexclusive manner implies that vanadate does not preferentially bind to the α-phosphate position of the NTP binding site, where the transition state is presumed to develop. It is also possible that vanadate binds to several sites in the active site of RT (including the α-phosphate position) with equal affinity, but in the presence of TMP it binds in the alternate positions.

There is incomplete antagonism which develops between vanadate and TMP suggesting that the preferred mode of binding for vanadate overlaps with the α-phosphate region of
TABLE VIII
Combined Inhibitor Studies with HIV-RT: Vanadate

<table>
<thead>
<tr>
<th>Ligand 1</th>
<th>Ligand 2</th>
<th>Interaction Constant (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V0₄⁻</td>
<td>PFA</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>V0₄⁻</td>
<td>thymidine</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>V0₄⁻</td>
<td>TMP</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>V0₄⁻</td>
<td>TDP</td>
<td>3.4 ± 1.0</td>
</tr>
</tbody>
</table>

* The interaction constants are reported as the average ± the standard deviation which were obtained from two separate experiments that each yielded 4 K values.

the substrate ATP binding site. It is also possible that vanadate binds to the 5'-phosphate position of the ATP binding site and the electrostatic repulsion inhibits the formation of the ATP-vanadate RT complex. Alternatively, the vanadate ion interacts preferably with a form of the enzyme that will not bind TDP. These results imply that vanadate interacts with various anionic binding sites on RT and does not preferentially interact with the 6-phosphate position of the ATP binding site. Despite its greater inhibitory potency compared to the phosphate ion, it appears not to inhibit as a transition state analog of the RT reaction.

2.4.1 (g) INHIBITION OF RT BY THYMIDINE DERIVATIVES WITH 5'-STERICANAL VARIATIONS.

In order to probe the interaction of anionic ligands at the triphosphate region of RT, thymidine derivatives with various 5'-substituents were tested for inhibition of the enzymes (Table IX). Thymidine was a very weak inhibitor of all three RTs, and increasing the number of 5'-phosphate groups causes an incremental drop in the IC₅₀ value for all enzymes. It is apparent that each of the three phosphates on the substrate develops significant interactions with the ATP binding region of RT.

It was interesting that the methylene analog of TDP was a better inhibitor than TDP for both AMV-RT and MLV-RT. The different bond lengths and angles in methylene diphosphate compared to pyrophosphate (Larsen et al., 1989) may result in a slightly different orientation of the phosphate groups which allows a stronger interaction with these RTs.

In order to investigate the importance of ionic interactions at the triphosphate binding region of RT, two thymidine derivatives with hydrophobic 5'-substituents were tested for inhibition of the enzyme. The 5'-tosyl derivative was a weak inhibitor of all three enzymes. However, the 5'-p-nitrophenoxyphosphate derivative of thymidine was a better inhibitor than TMP for all three RTs, suggesting that the nucleophilic nitro group promotes many interactions with the triphosphate binding site of RT. For HIV-RT however, it is possible that this compound may be binding to a secondary, non-catalytic ATP binding site described by others (Furman et al., 1991). Non-competitive inhibition of HIV-RT by NTPs which are complementary (Furman et al., 1991) and non-complementary (West et al., 1992) to the template present in the activity assay has been observed. The Kᵢ for inhibition by NTPs is several times greater than the Kᵢ for the substrate NTPs, and therefore the alternative binding site has a relatively low affinity for NTPs.

2.4.2 INTERACTION OF OP WITH HIV-RT

The initial studies with HIV-RT found that OP (0.5 mM)
was able to inhibit this enzyme by 50% in the presence of a
tenfold excess of added Mg\(^{2+}\) ions. The non-chelating analog
of OP, p-phenanthroline caused less than 10% inhibition of
polymerase activity at the same concentration. Thus the
inhibitory effects of OP on HIV-RT appeared to be due to its
ability to chelate a metal ion other than the substrate-
activating Mg\(^{2+}\) ion present in the assay.

2.4.2 (a) INHIBITION OF HIV-RT BY OP.

The interaction of a metal chelator such as OP with a
metalloprotein often involves two different processes. The
first process is usually rapid and reversible and involves
the formation of a ternary complex of the chelating agent
with the metal and enzyme, while the second is often time-
dependent and involves a dissociation of the chelate-bound
metal from the enzyme. In preliminary experiments it was
determined that both processes occurred during the
interaction of HIV-RT with OP at 37°C and the second process
could be delayed by reducing the temperature and minimizing
the period of exposure of the enzyme to OP. Hence it was
found that in the presence of OP at 22°C the activity of
HIV-RT was linear over a 10 minute period (Figure 5, inset),
indicating that inactivation was not occurring during the
assay. This finding allowed the separate investigation of
the inhibition and inactivation of this enzyme by OP. The

site of metal chelation during the rapid inhibition of
HIV-RT by OP was then characterized by looking at
competitiveness towards the substrate or the template-primer.
The inhibition of HIV-RT by OP was clearly non-competitive
with respect to the substrate NTP (Figure 5), and therefore
the binding site of OP does not overlap with that of the
substrate NTP.

However, when the concentration of template-primer was
varied, OP behaved in a manner which is not inconsistent with
a competitive type of inhibition, although the accuracy of the
data is not sufficient to rule out a non-competitive type
of inhibition (Figure 4). Therefore the metal chelator most
likely competes with the template-primer for binding to the
active site of HIV-RT. This implies that the metal is found
within the template-primer binding region, or the chelation
of the metal at another site on the enzyme changes the
affinity of the binding region for template-primer. These
results are consistent with the metal ion being located
within the active site of this RT, however it is important to
determine whether the putative metal is required for
structural integrity of the active site, if it participates
in the binding of the substrates or it is directly involved
in the catalytic process.

The results from these kinetic studies on the
interaction of OP with HIV-RT are somewhat different from
earlier studies done with AMV-RT. It was reported that CP is a non-competitive inhibitor of AMV-RT when either the substrate dNTP or the template-primer concentrations were varied (Poiesz et al., 1974). Although this indicates that CP may interact with the two enzymes differently, it is important to note that in the AMV-RT studies, the assays were carried out under different conditions. Those experiments were done at 37°C, a temperature where CP is known to inactivate the polymerase activity of this enzyme (Bald et al., 1974) while the HIV-1-RT assays done in this research were carried out at 22°C, where no inactivation of this enzyme occurs. It is therefore possible that the difference in the kinetic interactions of CP with the two RTs with respect to the template-primer could reflect this difference in assay conditions. The non-competitive character of the inhibition of AMV-RT by CP reported earlier (Poiesz et al., 1974) when the template-primer concentration is varied may be in part due to the occurrence of inactivation conditions.

It is interesting that interaction of CP with HIV-1-RT is kinetically similar to that previously reported for another DNA polymerase, mammalian terminal deoxynucleotidyl transferase (Chang & Bolund, 1970). CP inhibits this enzyme non-competitively with the dNTP substrate and competitively with an oligonucleotide initiator. Since this enzyme does not use a template, this suggests that the site of chelation is within the initiator binding site. If the function of the metal in this enzyme and in HIV-1-RT is similar, this would suggest that the bound metal is not involved in the binding of the template to the enzyme. Thus the putative metal ion of HIV-1-RT may be within the primer binding region. Experiments which could address this issue will be described in a later section.

2.4.2 (b) THE COMBINED EFFECTS OF Cu" AND CP ON HIV-1-RT

Previous studies on the mechanism of inhibition of various polymerases by CP have suggested that the reversible inhibition of polymerases is not due to the chelation of an essential metal ion at the enzyme active site (D'Aurora et al., 1978; Sigman et al., 1979) but to the formation of an (CP):Cu" complex which causes a rapid cleavage of the template-primer in the assay (Sigman et al., 1979). Thus it is important to ensure that this mechanism is not responsible for the rapid inhibition of RT observed with CP. To this end, inhibition studies of HIV-1-RT with CP and the cupric ion were done in the presence of 2 mM dithiothreitol, which is reportedly required to reduce the cupric ion to the cuprous ion (D'Aurora et al., 1977). Since the IC50 values for the inhibition of HIV-1-RT by CP (0.5 mM) and the cupric ion (10 mM) are at least two orders of magnitude larger than the reported IC50 value for the inhibition of POL I by the
(GP), Cu²⁺ complex (0.1 μM; D'Aurore et al, 1977; D'Aurore et al, 1978), the combination of GP and Cu⁺ should produce marked synergistic inhibition of HIV-1 RT if the specific complex is formed and it inhibits the polymerization reaction of HIV-1 RT with a similar potency as it does POL I. The median effect plot for the inhibition of HIV-1 RT by GP alone, Cu⁺ alone and the combination of Cu⁺ and GP is shown in Figure 7. The combination of GP and Cu⁺ exhibits a greatly reduced potency compared to either of the compounds alone. One explanation for this could be that the cupric and/or cuprous ions are to a large extent complexed with GP and the complexes are not inhibitors of the RT reaction, and the uncomplexed GP is responsible for the remaining inhibition observed. These results are in agreement with those of Massa & Falchuk (1984) which suggest that the mechanism of GP inhibition of polymerases is due to the chelation of a functional enzyme-bound metal ion.

2.4.2 (c) TIME-DEPENDENT INACTIVATION OF HIV-1 RT BY GP.

In order to investigate the occurrence of a time-dependent process in the interaction of GP with HIV-1 RT, the effect of the chelator on the stability of HIV-1 RT during prolonged incubation at both 0°C and 37°C was examined (Figure 8). The reversibility of the inhibition was tested by diluting the GP-enzyme mixture to yield a non-inhibitory

\[
CI = \frac{IC_{50, GP} + IC_{50, Cu} + IC_{50, GP \cdot Cu}}{IC_{50, GP} + IC_{50, Cu} + IC_{50, Cu \cdot GP}}
\]

A CI value of 1 indicates antagonism between two compounds for the inhibition of an enzyme. The value obtained from this plot was 3.6.

FIGURE 7: Median Effect Plot for the Inhibition of HIV-1 RT by GP and Cu⁺ Alone and in Combinations. Various concentrations of GP alone, the Cu⁺ ion alone, and a mixture of Cu⁺ and GP (a molar ratio of 0.3:1) were added to an activity assay prior to the addition of the enzyme. The combined effect was evaluated by determining the concentrations of each inhibitor present at the IC₅₀ of the mixture. The combination index (CI) was then determined according to the following equation:

A CI value of 1 indicates antagonism between two compounds for the inhibition of an enzyme. The value obtained from this plot was 3.6.

FIGURE 8: Time-dependent inactivation of HIV-1 RT by GP. HIV-1 RT was incubated for 30 min at 0°C with 0.5 μM GP (×) and at 37°C with 0.5 μM GP (×) or 0.5 μM GP (×). Aliquots were withdrawn at the times indicated, and assayed for activity as described in the Methods section. The level of GP in the polymerase assay was kept constant for all samples by adding GP (0.05 μM) directly to the polymerase assay for the enzyme aliquots incubated without GP. The curve obtained from incubation at 0°C in the absence of GP was identical to the one with GP and was omitted from the figure for clarity.
2.4.2 (C) THE EFFECT OF DIFFERENT LIGANDS ON THE INACTIVATION OF HIV-1-RT AND MLV-RT BY GP.

In order to further characterize the process of inactivation by GP, the effect of template-primer and other ligands on the inactivation of both HIV-1-RT and MLV-RT by GP was examined (Table X). The presence of excess levels of template-primer provided significant but incomplete protection against inactivation for both enzymes. Therefore, the binding of template-primer to both RTs causes a significant reduction in the rate of metal removal but does not eliminate it. These results are consistent with the kinetic studies indicating that GP interacts with the template-primer binding site.

The addition of saturating levels of TTP further increased the protection for both enzymes, with a more pronounced effect for HIV-1-RT. It was striking that the presence of TTP and template-primer provided almost complete protection of HIV-1-RT from the effects of GP.

The effect of the product pyrophosphate or its analog phosphonofumurate (PFA) was similar to that of TTP, in that these compounds were able to shield the enzyme from the effects of the chelator.

The protective effect of TTP, PFA, and PP, was an unexpected result and prompted an examination of the effect of these compounds on the stability of the enzymes in the concentration of GP before assaying for polymerase activity. At 0°C the enzyme was stable over the preincubation period of 30 min in the absence or presence of 0.5 mM GP. However, preincubation of the enzyme at 37°C with 0.5 mM GP resulted in a progressive inhibition of RT activity that was not reversed by tenfold dilution. This suggests that GP can inactivate HIV-1-RT in a time- and temperature-dependent manner and that this process may involve the dissociation of the chelate-bound metal from the enzyme. Previous studies on AMV-RT have shown that incubation with 1 mM GP at 37°C but not at 25°C brings about inactivation of the polymerase activity of this enzyme (Auld et al., 1974). Therefore, the inactivation of both dimeric RTs is not only time-dependent but is also heat-sensitive.

A similar type of inactivation was also found with MLV-RT. Incubation of this enzyme with 1 mM GP for 1 hr at 37°C brought about an 85-90% reduction in polymerase activity when assayed after a tenfold dilution of the GP. This RT is a monomeric enzyme that is only distantly related to the dimeric HIV-1-RT in the retroviral phylogenetic tree (Xiong & Eickbush, 1988, McClure et al., 1988). These results imply that a protein bound metal plays an essential role in the function of both monomer and dimeric RTs, and that the requirement of a metal for the polymerase activity of RT may have been conserved during the evolution of retroviruses.

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>template-primer</td>
<td>HIV-1-RT</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>TTP</td>
</tr>
<tr>
<td>+</td>
<td>PFA</td>
</tr>
<tr>
<td>+</td>
<td>PP</td>
</tr>
</tbody>
</table>

Enzyme aliquots were preincubated ± template-primer and ± second ligand for 10 min at 37°C. GP was added to start the 60 min inactivation period at 37°C. Aliquots were then diluted 10-fold into polymerase activity assay, and assayed as described in the Methods section.
absence of template-primer (Table XI). This was investigated to rule out the possibility that these active site ligands provided protection of the enzyme in the absence of template-primer. Surprisingly, these ligands did not provide any protection on their own. On the contrary, the rate of inactivation of HIV-RT was slightly enhanced in the presence of FFA or PFA. However, none of these ligands influenced the stability of either enzyme in the absence of OP. Therefore occupation of the binding sites for the substrate MTP, FFA or the product PP, hinders the ability of OP to either approach the metal or remove it from the enzyme only in the presence of template-primer. Since the RT reaction follows an ordered mechanism whereby template-primer first combines with the enzyme, the substrate MTP binds and then the product PP, is released (Hajmudar et al., 1988), it is possible that these ligands can not bind to RT in the absence of template-primer and therefore no protection from inactivation is observed. Alternatively the substrate MTP, and the product PP, or the inhibitor FFA bind to these MTP in a different mode in the absence of the template-primer, and this alternative mode of binding cannot afford protection from OP-mediated inactivation.

The results from the kinetic and protection studies both suggest that the site of metal chelation is within or very close to the template-primer binding region. However, the kinetic studies show that the substrate MTP binding site and that of OP do not overlap, while the protection studies indicate that TPP can delay the inactivation of HIV-RT by OP. One possible interpretation of these observations is that the binding of OP and TPP to HIV-RT is indeed mutually exclusive. The binding of TPP to the enzyme-template-primer complex would not directly hinder the level of OP binding but could induce a conformational change in the enzyme which makes it more difficult for OP to access the metal binding site or for the chelator to remove the metal from the enzyme.

This explanation is supported by recent studies on the mechanism of HIV-RT (Kati et al., 1992). Steady-state kinetic analysis of the polymerization reaction catalyzed by HIV-RT indicated that the binding of complementary MTP to the binary enzyme-template-primer complex changes the strength of binding of DNA to the enzyme. Specifically, the dissociation rate of the E-DNA (a 25/45-mer duplex DNA) complex was 0.2 s⁻¹ in the absence and 0.02 s⁻¹ in the presence of the complementary MTP (Kati et al., 1992). Since the primer was terminated with a deoxynucleoside, this change in the binding strength of DNA was not coincident with the chemical catalysis step. Thus the binding of MTP to the E-DNA complex inhibits tenfold the dissociation of DNA from the enzyme, presumably via a precatatalytic isomerization of the enzyme.

During the incubation of HIV-RT with OP in the present studies, the presence of template-primer would provide direct protection from inactivation by OP by physically occluding the chelator from its binding site. Inactivation would only occur when the template-primer dissociated from the active site, allowing OP to access the metal. The addition of TPP to the incubation would promote the conformational change described above, lead to reduced dissociation of the DNA-DNA from the active site and therefore reduce the exposure of the active site metal to OP.

The protective effect of both pyrophosphate and FFA may also be explained in a similar fashion. Unlike OP, neither of these compounds is a competitive inhibitor of HIV-RT with respect to the template-primer, and therefore these compounds would not likely antagonize the binding of OP to the enzyme. These two compounds may cause a conformational change similar to that with TPP which causes the template-primer to be bound tighter to the enzyme. The dissociation rate of DNA from HIV-RT in the presence of pyrophosphate was not measured in the above studies (Kati et al., 1992) and there is thus no experimental evidence for such an effect.

It is tempting to speculate that HIV-RT exhibits a kinetic mechanism similar to that for HIV-RT (Kati et al., 1992) which would explain the increased protection provided
by the substrate TTP, pyrophosphate and PFA. In the absence of any studies on this enzyme concerning the kinetic mechanisms, it is impossible to rule out that there are alternative reasons for the protection profile seen.

2.4.3 Reactivation of K75 and Pol I by Various Metals

OP has a high affinity for not only Zn⁺⁺ but for other metals such as Cu⁺⁺ and Co⁺⁺ and a moderate affinity for Mn⁺⁺ (Ellen, 1964), which is often used as the substrate activator for the MLV-WT activity assays. Therefore the observed inactivation of these enzymes by OP could be due to chelation and removal of a metal(s) other than zinc. For this reason, the ability of different metal ions to restore activity to the OP-inactivated polymerases was determined (Figure 9). These experiments were performed by incubating the enzymes in buffer with a high concentration of OP and then diluting tenfold into metal-containing buffer to allow reactivation. Since some metal ions are effective inhibitors of polymerases, the reactivation mixture was then diluted fivefold into the activity assay to further reduce the concentration of metal ions. Thus the final concentrations of OP and metal ions in the activity assay were 20 μM and 22 μM for HIV-1-WT respectively, and 10 μM and 11 μM for both MLV-WT and PolI respectively. The OP concentration in the activity assay was the same regardless of whether the samples had been preincubated in the presence or absence of OP.

When assayed in the presence of only the substrate-activating Mg⁺⁺ and low levels of OP which would serve to chelate any adventitious metal contaminants, the three enzymes incubated with OP exhibited 10-15% activity of the controls which were incubated without OP. This would indicate that for all three enzymes, a low level of polymerization is supported by Mg⁺⁺ alone.

None of the metals were able to restore 100% activity to any of the enzymes, most likely due to increased instability of the metal-free enzyme. Although zinc was capable of restoring function to all three polymerases, each enzyme had a different reactivation profile with the other metals. It should be noted that while the most apparent explanation for the inability of different metal ions to restore activity to each polymerase would be that the specific properties of the metal do not satisfy the requirements of the binding site of each enzyme, there is an alternative explanation that cannot be ruled out with the present data. It has been established that PolI and most likely other polymerases have multiple classes of divalent metal ion binding sites, some of which may be inhibitory (Slater et al, 1972; Mullen et al, 1990).

Thus during the two hour reactivation period in the presence
of 50 to 100 μM levels of specific metals, these metal ions could bind to the primary binding site and to some secondary metal binding sites. Although there was a tenfold dilution of the reactivation mixture into the activity assay, if these metals were strongly bound to the inhibitory binding sites this would result in minimal polymerase activity being expressed even if the metal ion bound to the primary metal binding site in a functional manner.

The reactivation profile for PolI was similar to that reported for PolII which had been inactivated by prolonged dialysis against 10 mM NP (Springgate et al., 1973). Zn²⁺ and Co²⁺ restored activity to 75% and 90% of original activity respectively, while Mn²⁺ restored just over 50% activity. The Cu²⁺ ion, however, restored little activity above that seen with no metal ions present during reactivation, with polymerase activity determined in the presence of high levels of Mg²⁺. Thus PolI appears to have at least one metal binding site with a metal preference of Co²⁺ > Zn²⁺ > Mn²⁺ > Mg²⁺.

HIV-1-RT displayed a different reactivation profile compared to PolI. It was surprising that copper was able to restore activity to HIV-1-RT, since this ion generally does not functionally substitute for zinc in metalloenzymes (Vallee & Galdes, 1984). Although copper is a better Lewis acid than zinc, it does not easily adopt the regular symmetry of coordination associated with the metal-binding site of zinc enzymes. One exception to this is the substitution of copper for zinc in the KF binding subunit of DNA polymerase from E. coli (Matterzi & Wu, 1982a). The copper-substituted enzyme has 17% of the activity of the zinc enzyme. Therefore the putative zinc ion in HIV-1-RT may have a similar type of co-ordination or function as that found in E. coli RNA polymerase.

Cobalt generally substitutes for zinc in enzymes (Vallee & Galdes, 1984) and therefore it was surprising that this metal was only able to restore activity to PolI and not HIV-1-RT. Cobalt apparently does not restore activity to zinc-free AMV-RT (Poiesz et al., 1974), suggesting a similar binding site may exist in these two viral ribozymes. This metal is not as good a Lewis acid as zinc, but it will readily assume the unusual coordination spheres found in zinc enzymes.

It is difficult to explain why partial activity was obtained with the Mn²⁺-substituted forms of the monomeric polymerases M1-RT and Pol I but not HIV-1-RT. Previous studies on the reactivation of Zn²⁺-free AMV-RT by dialysis have also indicated that Mn²⁺ was unable to restore activity to this enzyme (Auld et al., 1974). This would suggest that the dimeric RTs have a metal binding site with slightly different specificities compared with the monomeric polymerase and the monomeric RT.

It is possible that manganese is able to restore activity to HIV-1-RT, but that the rate of reactivation for this enzyme is much slower than Pol I and M1-RT, and therefore little activity is seen after the two hour reactivation period. This type of phenomenon is seen with the reactivation of the metalloenzyme carboxyl anhydrase (Billot et al., 1978). The formation rate constant at pH 7 for the reaction of apoaplastic anhydrase with Mn²⁺ is three orders of magnitude smaller than that for Zn²⁺.

In the presence of millimolar levels of Mn²⁺, Mn²⁺ was found to inhibit the polymerase activity of HIV-1-RT. In order to rule out the possibility that the residual levels of Mn²⁺ in the activity assay of HIV-1-RT were sufficient to inhibit the enzyme, it was determined that the IC₅₀ for the inhibition of HIV-1-RT by the Mn²⁺ ion was 0.10 mM, nine-fold higher than the level in the assay. This would result in minimal inhibition of the enzyme.

The different reactivation profiles obtained may be explained by differences in the geometric requirements of the metal binding site for each polymerase, and thus the environment of the essential metal is significantly different for each of the polymerases.

It is important to note that all of the viral enzymes used in this research were cloned and then produced in E. coli. Since de novo synthesis of the proteins occurred in bacterial cells and not in the proper host animal cells, it is possible that there are some differences between the naturally produced and the cloned enzymes. One of these may be the identity of the associated metal ion. Therefore the reactivation profile of each enzyme yields information on the specificity of the metal binding site but does not yield information on the identity of the in vivo metal(s).

2.4.2 (f) Comparison of the Native and Activated Forms of HIV-1-RT

In order to further probe the involvement of the metal in the function of HIV-1-RT, the binding of known Mg²⁺ ligands to the Cu²⁺ and Zn²⁺-activated enzymes as well as the native recombinant enzyme was investigated. Three inhibitors, each with a different structure and presumed mode of inhibition, were used for initial inhibition studies with native HIV-1-RT and its Zn²⁺- and Cu²⁺-activated enzymes (Table XII). The Cu²⁺-activated enzyme had different sensitivities to these inhibitors, and thus further studies with additional compounds were done. However it was of obvious importance to ensure that the observed differences were due to the presence of a different metal rather than an artifact of the inactivation-reactivation procedure. The control experiments found that the native and Zn²⁺-activated enzymes displayed similar sensitivities to PFA, TDP and formohydroxamate (Table
Table XII
The inhibition of native and reactivated forms of HIV1-RT

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Native % Activity</th>
<th>Activated % Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFA</td>
<td>0.35</td>
<td>52</td>
<td>60</td>
</tr>
<tr>
<td>TDP</td>
<td>120</td>
<td>48</td>
<td>28</td>
</tr>
<tr>
<td>formo-</td>
<td>4100</td>
<td>53</td>
<td>90</td>
</tr>
<tr>
<td>hydroxamate</td>
<td></td>
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</tr>
</tbody>
</table>

2.4.2 (g) INHIBITION OF COPPER-ACTIVATED HIV1-RT.

The level of activity of the copper-activated HIV1-RT obtained in the presence of a variety of known RT inhibitors at their IC\textsubscript{50} concentrations for the native enzyme is presented in Table XIII. The fact that copper substitution changed the level of inhibition obtained with all of the ligands implies that the conformation of the metal-binding site plays an integral part in the binding of ligands to and the polymerase function of HIV1-RT.

The copper-activated enzyme was slightly less susceptible than the native enzyme to inhibition by the anionic inhibitors PFA, carbonyldiiphosphonate and oxalate. The similar effect of metal substitution on the inhibition by these compounds is in agreement with the multiple inhibitor studies on HIV1-RT showing that these compounds bind in a mutually exclusive manner to the enzyme. The very modest effect of copper substitution suggests that the binding of pyrophosphate and its analog, PFA, is not largely dependent on the nature of the metal ion. If the interaction of pyrophosphate with the active site of HIV1-RT involved direct co-ordination with the metal ion, a larger change in affinity would be expected to occur with copper substitution. Hence the binding of this reaction product may be based on ionic interactions with basic residues at the active site. This is in agreement with the idea that the cation used to activate the substrate ATP (M\textsuperscript{+} in the case of HIV1-RT) may serve to chelate pyrophosphate within the active site of HIV1-RT.

The copper enzyme has a greater affinity for the nucleoside thymidine diphosphate (Table XIII) than the native enzyme, and therefore the copper ion may promote additional interactions with this nucleoside. Since there is little change in the binding of pyrophosphate analogs, these additional contacts must occur through the thymine base or ribose substituents of TDP.

Evidence has suggested that Cu\textsuperscript{2+} is able to co-ordinate with the purine ring of ATP in solution while Zn\textsuperscript{2+}, Mg\textsuperscript{2+} and Mg\textsuperscript{2+} do not (Scheiner et al., 1964; Cohen & Hughes, 1962). It is possible that unnatural interactions develop between the Cu\textsuperscript{2+} ion and the pyrimidine ring of TDP which thereby enhance the affinity of the Cu\textsuperscript{2+}-activated enzyme for this nucleoside relative to the native enzyme. Under the assumption that TDP binds to the substrate binding site, this implies that the nature of the metal ion in HIV1-RT could play some role in binding of the substrate. This is not in agreement with the kinetic studies on the inhibition of HIV1-RT by DMS that
indicate the binding site for this metal chelator does not overlap with that of the TTP substrate. In addition, the reactivation studies indicated that the Cu"+ and In"+ activated enzyme displayed similar levels of polymerase activity when TTP is present at the Ks levels for the native enzyme. An alteration of the binding constant for the substrate would therefore have been reflected in altered levels of polymerase activity for the Cu"+ substituted enzyme. Since the metal binding site is not within the NTP binding site of the enzyme, it seems most likely that abnormal interactions between the Cu"+ ion and the ligand TTP occur. Nonetheless, it would be of interest to investigate the activity of the Cu"+ reactivated enzyme on different homopolymeric templates and the corresponding substrates to determine the effects on the polymerization of the triphosphate forms of adenosine, cytosine and guanosine.

Inhibition by the vanadate ion and two small hydroxamate compounds was affected by copper substitution to a much greater extent than the three pyrophosphate analogs, suggesting that the putative metal ion has a greater role in the binding of these compounds to HIV-1-RT. It is possible that these three compounds do not bind to HIV-1-RT as analogs of the product pyrophosphate but that they bind to a site(s) that is affected to a greater extent by the nature of the essential metal ion. This is supported by the fact that combined inhibitor studies with HIV-1-RT have shown that the vanadate ion had a different mode of inhibition of this enzyme than the pyrophosphate analog PFA. It is possible that the two hydroxamate compounds and vanadate have some type of a direct interaction with the active site metal ion(s).

2.5 DISCUSSION

A variety of methods were used in this study to further investigate the active site of RT. The main goal of this research was to study the active sites of various RNAs with different methods in order to gain further understanding of the binding and catalytic functions of these enzymes.

2.5.1 THE ANIONIC BINDING SITE OF RTs

The structure-activity studies with small anionic inhibitors gave some insight into the characteristics required for effective inhibition of RTs. Since these compounds are presumed to bind to the pyrophosphate binding site of the enzyme, it is not surprising that a charged phosphate or phosphonate moiety is required. In addition, binding is optimal when this group is accompanied by an adjacent negatively charged group. The compounds PFA and hypophosphonate satisfy these requirements and are among the best inhibitors of the polymerases tested. The general trends of the structure-activity study indicate that the bacterial polymerase was the least sensitive to the best anionic inhibitors, AMV-RT and HCV-RT were more effectively inhibited and HIV-1-RT was the most sensitive to them.

Since it was clear that PFA was the optimal anionic inhibitor of the RTs, the combined inhibitor studies were performed in order to investigate the binding interactions of PFA and other active site ligands to HIV-1-RT. The results supported the previous contention that PFA acts as a pyrophosphate analog (Vraag & Oberry, 1986). The combined inhibitor studies with PFA and various phosphorylated forms of thymidine suggest that PFA preferentially interacts with the 5'- and 4'-phosphates regions of the NTP binding site. This is consistent with site-directed mutagenesis studies on HIV-1-RT which have demonstrated that mutations that alter the Ks for the substrate TTP also alter the Ks for PFA (Lowe et al., 1991). The exact mode of interaction of PFA with HIV-1-RT is not as straightforward as implied by these results. Since it interacts with HIV-1-RT in a mutually exclusive manner with respect to both the substrate and the non-competitive hydrophobic pyridinones inhibitors (Goldman et al., 1992), PFA inhibits a unique form of the enzyme.

It would therefore be of interest to design a nucleoside compound with a PFA moiety in the 5' region of the ribose group. This type of an adduct may provide additional specificity for the inhibition of RT with less toxicity compared to PFA, since several of the side effects of PFA are presumably due to its similarity to phosphate and pyrophosphate. Such a compound could take advantage of the binding energy available to PFA and the substrate NTP and could be a more potent RT inhibitor than PFA alone. The
results from this study would suggest that the PFA moiety of such an adduct would have to extend into the 5'- and 4'-phosphate regions of the substrate binding site for effective interaction.

Effective antiviral activity requires transport of the inhibitor across the host cell membrane before interaction with the target viral enzyme. While a PFA-nucleoside adduct would be charged, this may not necessarily preclude membrane transport. This is indicated by the fact that the charged acyclic nucleoside derivatives FMEA and FFMEA (Figure 3), which are inhibitors of HTLV-RT in their diphosphorylated form exhibit effective antiviral activity (De Clercq et al., 1986).

It is clear that PFA represents a near optimal inhibitor for binding to the pyrophosphate region of RT and from the results presented in this study it is unlikely that structural variation of this compound will result in a tighter binding analog.

2.5.2 THE INTERACTION OF A METAL CHELATOR WITH RTs

The sensitivity of several RTs and DNA polymerases to OP has been known for quite some time, but its mode of inhibition of these enzymes has been a subject of controversy (Sigman et al., 1979; Manus & Falenchuk, 1981). The evidence presented here suggests that the interaction of HTLV-RT with OP appears to involve two processes; a rapid and reversible inhibition and a time-dependent inactivation of the enzyme. These processes appear to be associated with the cleavage and removal of an essential metal(s) from this enzyme. The reactivation studies presented here suggest that zinc is required for the function of HTLV-RT and two other RTs, which is in agreement with previous investigations into the metal content of RTs (including AMV-RT and HLV-RT) and its correlation with polymerase activity (Auld et al., 1974; Auld et al., 1975). However, for HTLV-RT it is important to note that the inhibition of this enzyme by a chelating agent is not conclusive evidence that it is a metalloenzyme; this has yet to be definitively demonstrated by sensitive metal analysis of this enzyme.

2.5.3 ACTIVE SITE ZINC LIGANDS

The relative ease with which the metal is removed from the enzyme suggests that the metal is easily accessible and therefore may have a catalytic rather than structural role. The catalytic zinc associated with enzymes has three protein ligands and water as the fourth ligand which allows it to participate in the catalyzed reaction (Vallee & Auld, 1980). On the other hand four protein ligands co-ordinate structural zinc atoms in enzymes, and they are therefore much more difficult to remove.

Previous analysis of active site zinc ligands have shown that the residues involved in co-ordinating the metal are strictly conserved within each family of enzymes (Vallee & Auld, 1990). Under the assumption that all RTs are zinc enzymes, the residues involved in co-ordination of this metal should be conserved. Cysteine residues are common zinc ligands (Vallee & Auld, 1990) but there are no conserved cysteine residues within the RT family (Xiong & Elrod-Erickson, 1988). In addition it is known that cysteine residues are not involved in metal binding of HIV-RT, since substitution of the two cysteine residues of this enzyme with serine has no effect on the ability of OP to inhibit the polymerase reaction (Loya et al., 1992). A review of the very few residues strictly conserved amongst RTs (Xiong & Elrod-Erickson, 1988) points to the four conserved aspartate residues as potential candidates as ligands for the essential metal ion(s). Three of these are absolutely essential for HIV-RT polymerase activity (Lowe et al., 1991), and it is possible that part of their essential function is to co-ordinate an essential metal ion.

The lack of conserved cysteine or histidine residues in HIV-RT and the presence of the conserved aspartate residues within the active site (Kohlschütz et al., 1992) suggests that the putative metal binding site may not be a Zn" specific site, but that Zn" is one of the metal ions that it will bind with significant affinity. One example of a metal binding site composed exclusively of oxygen ligands donated by active site carboxyl groups that prefers Zn" over Mg" or Mn" is discussed in section 2.5.7.

2.5.4 METAL LOCATION IN RT

The present study gives some insight into the location of the active site metal in HIV-RT and the results are consistent with the possibility that the essential aspartate residues may be potential ligands for this metal. The competitive nature of the rapid inhibition of HIV-RT by OP with respect to the template-primer indicated that OP and the template-primer compete for the same binding site on HIV-RT. This suggests that the metal binding site is either within the template-primer binding region, or it is close enough such that the OP molecule extends into the region and causes steric hindrance to the binding of the template-primer. The results of the protection studies supported this observation and are consistent with the possibility that the essential metal may be involved in co-ordinating the primer terminus.

In addition, the recently determined crystal structure of HIV-RT suggests that the putative essential metal ion(s) is in close proximity to the template-primer binding region (Kohlschütz et al., 1992). In this enzyme, three catalytically essential aspartate residues form a tripod of
phosphoryl transfer mechanisms. The two step reaction involves the formation of a phosphoester intermediate and its subsequent dephosphorylation. In the first step of the reaction, the A site zinc forms a bond with the ester oxygen (ROP) thus activating the leaving group (RO'). The B site zinc coordinates a second phosphate oxygen. The oxygen of an active site serine residue then forms a new ester bond with the phosphorus to produce a phospho-ester enzyme intermediate. After the departure of the leaving group, the A site zinc then coordinates a water molecule. Metal-induced proton dissociation generates an hydroxide ion which is the nucleophile in the second step of the hydrolysis reaction. In this second reaction, the B site zinc ion activates the leaving group by co-ordination of the ester oxygen of the phosphoester intermediate. Thus the zinc ion functions in activation of the leaving group in both steps and of the attacking nucleophile in the second step of the alkaline phosphatase reaction.

It is possible that the putative zinc ion(s) in ROP perform one or more functions in phosphoryl transfer similar to that seen with alkaline phosphatase. One of the functions would then be activation of the attacking nucleophile by promoting ionization of the 3'-hydroxyl of the primer terminus. Alternatively, co-ordination of the α-α-β ester oxygen of the substrate ATP by the zinc ion(s) would serve to activate the leaving group, pyrophosphate.
appear that these two specific RNA and protein catalysts may have both evolved to promote their respective phosphoribose-nucleotidyl transfer reactions by binding at least two different divalent metal ions which serve different functions in order to enhance the nucleophilic attack of a 3′ hydroxyl group of a ribose moiety on a nucleotidyl α-phosphoryl group and the ensuing reaction.

2.5.7 THE METAL IONS OF 3′-5′ EXONUCLEASE FROM POL I

The 3′-5′ editing exonuclease activity of Pol I resides on a structural domain which is distinct from that of the polymerase domain. Crystal structure studies on the exonuclease domain have indicated that there are two metal ions bound to the active site. They are separated by 3.9 Å and are both bound by carboxylate groups (Beece & Steitz, 1991). Site A metal ion is pentacoordinate, utilizing the carboxylate groups of Asp555, Glu537 and Asp501, the 5′-phosphate of the product GMP and a water molecule as ligands. A second weakly bound metal ion (site B) is observed only in the presence of GMP, and shares its only protein ligand (Asp555) with metal A (Beece & Steitz, 1991). In addition it is coordinated by two of the 5′-phosphate oxygens of the product GMP and three water molecules to form octahedral coordination. Although the identity of the two metal ions in the in vivo reaction remains to be established,

in the presence of 1 mM Zn" and 20 mM Mg" the crystal structure indicated that Zn" occupies site A and the site B metal ion is Mg" (Beece & Steitz, 1991). This type of coordination of Zn" represents one of the few examples in the literature whereby active site carboxylate residues contribute the only protein ligands to the metal.

On the basis of this complex a two metal ion phosphoryl transfer mechanism has been proposed (Beece & Steitz, 1991). In this mechanism, metal ion A is positioned to form the formation of a hydroxide ion from a bound water molecule, thereby acting as a Lewis acid. The attacking hydroxide ion is properly oriented by a nearby tyrosine residue and the second carboxyl oxygen of Glu537 (one carboxyl oxygen serves as a metal ligand). Thus the function of the zinc ion in this enzyme may be similar to that postulated for several other metalloenzymes such as thermolysin (Matthews, 1988) and carboxypeptidase A (Kim & Lipscomb, 1990) where it causes nucleophilic activation of a bound water molecule.

The metal ion at site B is appropriately positioned to stabilize the pentacoordinate transition state. In the model-built pentacoordinate intermediate (Beece & Steitz, 1991) metal ion B interacts with both an equatorial phosphate oxygen and the axial oxygen which is opposite to that of the incoming hydroxide ion, and therefore could also help to activate the leaving group.

2.5.8 A PROPOSED TWO METAL ION MECHANISM FOR HIV-1 RT

In a fashion similar to the above mechanisms for the exonuclease activity of Pol I, it is possible that a somewhat analogous two metal ion mechanism exists for HIV-1 RT. A schematic model of this is presented in Figure 10. The first metal ion (presumably Zn" or Mg") would be tightly bound and tetrahedrally coordinated. One or more of the side chains of the tripod of conserved aspartates within the active site of HIV-1 RT (Kohlsbaek et al, 1992) could contribute three protein ligands for this ion while the fourth ligand would be provided by the 3′ hydroxyl group of the growing primer terminus. This tetrahedral co-ordination would explain why zinc is the preferred metal ion for reactivation of the OP-inactivated RT (magnesium tends to be co-ordinated in an octahedral manner). While the main function of the zinc ion would be activation of the 3′-hydroxyl group of the primer terminus, it could also coordinate and thereby stabilize an α-phosphate oxygen of the pentacoordinate transition state/intermediate.

The second metal ion in the proposed mechanism would be provided by the divalent substrate activator (Mg" or Mn") and would largely be co-ordinated by the substrate NDP. Since the three conserved asparagine residues are the only active site residues in HIV-1 RT that are potential metal ligands, it is proposed that this ion shares one of the
chelator CP with respect to the substrate NTP and the competitive inhibition observed with respect to the template-primer would support the above proposed role for this ion in co-ordinating the 3'-hydroxyl of the primer.

2.5.9 CONCLUSIONS

The present studies which centered on the structure-activity relationships for anionic inhibitors of RT have yielded some useful information and shown that alteration in the structure of the well-known inhibitor PFA generally leads to a decrease in potency of inhibition of RT. The combined inhibitor studies allowed a more detailed investigation of the mode of interaction of PFA with RT, yielding information which may be useful in the design of effective RT inhibitors that incorporate the structure of PFA and other known inhibitors that interact with adjacent sites together into one molecule.

Finally, the present work has investigated the interaction of a metal chelator with the active site of RT and given some insight to the location of its binding site relative to known active site ligands, but a greater understanding of the precise role of an active site metal ion or ions in nucleotidyl polymerisation by this enzyme will require further detailed experimentation.

3. ASPARAGINE TRANSAMINASE

3.1 INTRODUCTION

The enzymes of pyrimidine pathways play an important role in cell metabolism and proliferation by providing some of the precursors for the biosynthesis of nucleic acids. Aspartate transcarbamoylase (ATCase) catalyses the first unique step in pyrimidine biosynthesis, where carbamyl phosphate reacts with L-aspartate to form N-carbamoyl-L-aspartate and phosphate. It is one of the nucleotide biosynthetic enzymes found in elevated levels in several rapidly proliferating tissues including hepatomas (Elford, 1970) and brain tumors (Kedani et al., 1987) and is thus a target for the chemotherapy of cancer.

3.1.1 ATCase: A MODEL REGULATORY ENZYME

The structure and function of the enzyme from E. coli has been thoroughly investigated since it is a model regulatory enzyme that exhibits both heterotropic and homotropic interactions (Gerhart & Pardee, 1962; Betheh et al., 1969). Positive co-operativity, in the form of sigmoidal saturation curves, is seen in the presence of either L-aspartate or carbamoyl phosphate. This homotropic regulation is generally felt to be the result of a substrate induced configurational
change which causes the enzyme to undergo a conversion from the unliganded "E" state to the more highly active "S" state (Schachman, 1968). This allows a much greater activity response to small changes in substrate concentration.

The purified enzyme is inhibited by cytidine triphosphate (CTP); an end product of the pyrimidine synthetic pathway. Adenosine triphosphate (ATP), a product of the parallel pathway of purine synthesis, activates the enzyme to help maintain a balance between the two types of bases required for DNA synthesis. Therefore ATCase is subject to both parallel activation and feedback inhibition.

3.1.2 GENERAL DESCRIPTION OF THE ATCase MOLECULE

Native ATCase from E. coli is a highly structured dodecameric enzyme. The holoenzyme designated $\alpha_4 \beta_4$ (Chan & Mort, 1973) can be dissociated by mercurials into two catalytic and three regulatory subunits (Gerhart & Schachman, 1965). The catalytic subunit ($\alpha_4$) is a trimeric aggregate of 34K chains, while the regulatory subunit ($\beta_4$) is a dimer of 17 KD peptides with two associated zinc ions. Therefore the equation for dissociation is $\alpha_4 \beta_4 = 2 \alpha + 3 \beta$. The isolated catalytic subunit is insensitive to allosteric effectors and displays simple hyperbolic substrate saturation profiles. Both the maximum velocity ($V_{max}$) and the Michaelis constant ($K_m$) of the catalytic subunit are greater than the corresponding values for the holoenzyme. The regulatory dimer binds either CTP or ATP and is catalytically inactive. When the mercurial is removed from the dissociated catalytic and regulatory subunits, they spontaneously reassociate to form an aggregate similar to native ATCase (Gerhart & Schachman, 1965).

3.1.3 GENETIC CONTROL OF ATCase LEVELS

The level of ATCase within a cell is controlled by a repression mechanism. In the presence of high levels of uracil, the transcription of the gene for ATCase is inhibited along with that of the four other enzymes in the pyrimidine synthetic pathway. This finding led to the development of a purification procedure that allowed the isolation of large quantities of ATCase from an E. coli mutant (Gerhart & Holoubek, 1967). This mutant is diploid in the region containing the gene for ATCase, and it requires uracil for growth since it lacks orotidylic decarboxylase, an enzyme in the pyrimidine synthetic pathway. In the presence of uracil, the bacteria exhibit rapid cell growth and the level of ATCase is reduced by a repression mechanism. Upon removal of uracil from the media, ATCase synthesis is derepressed and large quantities of the enzyme are formed, accounting up to 4% of the total protein in this bacterium. This procedure has allowed extensive investigation of E. coli ATCase with numerous methods which require significant quantities of the protein of interest.

3.1.4 COMPARISON OF MAMMALIAN AND BACTERIAL ATCase

The enzyme from E. coli is often used as a model for studies directed towards the inhibition of the mammalian enzyme. It is therefore of interest to compare the enzymatic and genetic organization of ATCase from bacteria, and from eukaryotes. In differentiating eukaryotes from prokaryotes, it is proposed that the prokaryote ATCase is associated with a trimer of 120 KD which is similar to the E. coli catalytic subunit in structural features and kinetic parameters (Major et al., 1989). Comparative molecular modelling of the mammalian enzyme based on the coordinates of the E. coli enzyme has indicated that the configuration of the active site residues is virtually identical in the mammalian and bacterial enzymes (Scully & Evans, 1991). Thus the bacterial enzyme is a suitable model for the mammalian enzyme.

3.1.5 DOMAIN STRUCTURE OF ATCase

The structure of E. coli ATCase has been solved to a resolution of 2.9 Å (Kruse et al., 1985) and has confirmed that both the catalytic and regulatory peptides of ATCase are composed of two discrete tertiary domains. The first 97 residues of the regulatory subunit comprises the allosteric domain which is responsible for the binding of the nucleotide effectors and the interactions between regulatory subunits. The rest of the regulatory subunit is the zinc domain, which is involved in catalytic regulatory subunit interactions and contains a region with four cysteines that bind zinc. The first 150 residues of the catalytic polypeptide is the polar or carbamoyl phosphate domain and is responsible for binding of this substrate. Residues 154 to 300 of this polypeptide represents the equatorial or aspartate domain.

3.1.6 THE T-R TRANSITION

The changes in structure and enzymatic properties of E. coli ATCase upon the binding of substrate ligands or nucleotide effectors are consistent with the two-state model described by Monod (Monod et al., 1965). In this model, the holoenzyme exists in two distinct forms, T and R, which have differing activity, ligand affinity and conformation. The T-state predominates in the unliganded ATCase, and this form binds the negative allosteric effector CTP more strongly and
has a low affinity for the substrates. The R-state on the other hand has a high affinity for active-site ligands and the activator ATP. The structure of the enzyme complexed with the bisubstrate analog, [N-(phosphonomethyl)-L-aspartate (PALA) has been determined and is thought to represent the R state (Krause et al., 1987).

3.1.7 ORDERED MECHANISM

Steady state kinetic analysis of the reaction catalyzed by ATCase have indicated that the binding of substrates by the catalytic subunit is ordered, with carbamoyl phosphate binding first, aspartate binding second, followed by the dissociation of carbamoyl-aspartate and then phosphate (Porter et al., 1969). A small conformational change occurs upon carbamoyl phosphate binding while a large change occurs upon L-aspartate binding. This was based on product inhibition studies, where carbamoyl-L-aspartate is a noncompetitive inhibitor versus carbamoyl phosphate but is competitive with respect to L-aspartate (Porter et al., 1969). Phosphate was reported as a competitive inhibitor when carbamoyl phosphate is the varied substrate but noncompetitive against L-aspartate. This interpretation was supported by the fact that the bisubstrate analog PALA is a noncompetitive inhibitor versus L-aspartate but competitive when carbamoyl phosphate concentration is varied (Collins & Stark, 1971). Recent isotope trapping studies have indicated that the kinetic mechanism of ATCase is indeed steady-state ordered with some random character (Turnbull et al., 1992; Parmantier et al., 1992).

3.1.8 CATALYTIC MECHANISM

The reaction catalyzed by ATCase is thought to involve nucleophilic attack of the amino group of aspartate on the carbonyl carbon of carbamoyl phosphate and the formation of a tetrahedral transition state with the development of a negative charge on the carbonyl oxygen of carbamoyl phosphate (Figure 11). Kinetic isotope experiments done with 13C or 15N labelled carbamoyl phosphate indicate the presence of a step in the reaction in which bonding to the anhydride oxygen is unchanged while bonding to the carbonyl carbon of carbamoyl phosphate is altered, and that this step is important in determining the observed rate of reaction (Stark, 1971). This finding is consistent with the formation of a tetrahedral intermediate.

There are several possible ways in which the enzyme could promote the transcarbamoylation reaction. Proton extraction from the amino group of aspartate by an active site residue would enhance nucleophilic attack on the carbon of carbamoyl phosphate by general base catalysis. The enzyme could also promote the reaction by polarizing the carbonyl group to make the carbon more susceptible to nucleophilic attack by general acid catalysis. In addition protonation of the phosphate oxygen of carbamoyl phosphate by an acidic group on the enzyme would render the phosphate a better leaving group. The possibility of both types of catalysis is supported by the fact that the pH dependence of the kinetic parameters for catalysis indicate that at least four ionizing amino acid residues are involved in substrate binding and catalysis (Turnbull et al., 1992).

In the first model for the catalytic mechanism of ATCase proposed by Stark (Collins & Stark, 1969; Porter et al., 1969) the mechanical compression of the two substrates was emphasized as the major mechanism for enhancement of the transcarbamoylation reaction. A subsequent analysis of the crystal structure of the enzyme complexed with PALA indicated that protein bridges between the phosphate of carbamoyl phosphate and the α-carboxylate of aspartate by both Lys84 and Arg105 could promote the proper orientation and proximity of the two reactants to enhance the reaction in the absence of compression by the enzyme.

3.1.9 ACTIVE SITE RESIDUES

Site-directed mutagenesis studies in combination with the crystal structure of ATCase complexed with either PALA (Krause, K.L. et al., 1987) or a combination of carbamoyl and...
phosphate and succinate (Gouaux & Lipkowitz, 1986) have clearly indicated the potential residues involved in the binding of substrates and in the catalytic mechanism of ATCase.

3.1.9 (a) ASCARCYSTE BANDING

Initial studies with competitive inhibitors outlined the steric restrictions for the binding of aspartate (Porter et al., 1969; Davies et al., 1970) and indicated that a four carbon diacarbonylic acid moiety was required for binding. The crystal structure of the complex with PABA confirmed the cis conformation (Krause et al., 1987) and indicated that an extensive network of bonds at the active site allow ATCase significant selectivity that prevents carboxylation of other amino acids within the cell.

3.1.9 (b) CARBONYL PHOSPHATE BINDING

The phosphate moiety of PABA bound to ATCase makes extensive interactions with many different residues of the phosphate binding region of the enzyme. These multiple interactions would explain the absolute requirement of a phosphate moiety for binding to this region of the active site.

The carbonyl group of carbamoyl phosphate appears to interact with three residues, based on the crystal structure of ATCase liganded with PABA (Krause et al., 1986). Thus Arg105, His134 and Thr55 were implicated in the polarization of the carbonyl group and in stabilizing the subsequent tetrahedral intermediate. An absolutely critical role for His134 was ruled out by the finding that the mutant generated by replacement of this residue with alanine retained 54% of the wild-type activity (Rooney et al., 1988). Heavy atom isotope studies of the mutant indicate that His134 does play a part in the chemistry of the reaction since the formation of the tetrahedral intermediate is rate-limiting for the mutant enzyme (Waldrop et al., 1992). This residue has several roles in binding and catalysis since the alanine mutant has a decreased affinity for both substrate and exhibits a random kinetic mechanism.

The contribution of Thr55 in binding and catalysis has been investigated by site-directed mutagenesis. Conversion of Thr55 which interacts with both the carbonyl and phosphate moieties of carbamoyl phosphate to alanine causes a fivefold reduction of maximal activity (Kuo & Kentzias, 1989) and a large decrease in binding affinity for carbamoyl phosphate but not for aspartate. Thus this residue is important but not absolutely critical for catalysis. It was surprising, however, that the carbon isotope effect studies with the mutant enzyme suggest that this residue is not involved in stabilizing the oxygenation in the transition state (Waldrop et al., 1992).

The remaining candidate residues for interaction with the oxygen of the tetrahedral intermediate are Arg 105 and Arg 54. Mutation of the former residue to alanine decreased the maximum velocity by 4 orders of magnitude while the binding of both substrates remains unaltered (Stebbins et al., 1989), implying it may have a critical catalytic role in the transcarboxylation reaction.

3.1.10 (b) ASCARCYSTE INHIBITORS

3.1.10 (a) ASCARCYSTE ANALOGS

Early studies on the catalytic subunit of ATCase indicated that the best inhibitors that were competitive with L-aspartate had a four carbon diacarbonylate moiety in the cis conformation (Porter et al., 1969). Succinate was found to be a good inhibitor and the introduction of an α-substituent caused decreased binding affinity, as evidenced by the fact that even the natural substrate L-aspartate had a much larger dissociation constant than succinate (Porter et al., 1969). There are definite stereospecific restrictions at the aspartate binding site since a second substituent added at either the α- or β-three positions results in dramatically reduced binding but erythro-β-hydroxy-L-aspartate is a good substrate (Davies et al., 1970). The relative inability of ATCase to catalyze the carboxylation of other amino acids with similarity to aspartate suggests that the α-carboxylate

of aspartate supplies critical interactions with the active site.

3.1.10 (b) CARBAMOYL PHOSPHATE ANALOGS

Many compounds with a phosphate or phosphonate moiety inhibit the catalytic subunit indicating the phosphate binding site is most likely close to the surface of the protein. Since acetyl phosphate is an alternative substrate the amino group is not required for activity. Both phosphonoacetate and phosphonoformate are good inhibitors of the enzyme (Dennis et al., 1986; Porter et al., 1969). It is presumed that the mode of binding of these compounds puts the phosphate in the phosphate binding region and the second anionic substituent interacts with the carbamoyl binding region of the enzyme. This would indicate the preference for a negative charge within this region. This is in agreement with the crystal structure of the enzyme complexed with PABA whereby the carbonyl group of the inhibitor interacts with arginine, histidine and threonine side groups (Krause et al., 1987).

3.1.10 (c) BIOSUBSTRATE ANALOGS

Several inhibitors which incorporate the structural features of both substrates have been synthesized and tested for inhibition of ATCase. One of the first compounds tested,
PALA (Figure 13), is still the best inhibitor reported to date (Collins & Stark, 1971) and represents a putative intermediate where the amino group of aspartate has formed a bond with the carbonyl carbon of carboxamidophosphate prior to the loss of phosphate. In PALA the amide oxygen of the substrate carboxamidophosphate is replaced with a methylene group and the amino group of carboxamidophosphate is absent. The compound with a methylene group replacing the amino group of PALA is a tenfold less effective inhibitor (Sawyer et al., 1974), suggesting that the presence of an amide nitrogen or the polarizability of the adjacent carbonyl group are important determinants of inhibitor affinity. The amide nitrogen would presumably increase the polarizability of the carbonyl group compared to a methylene moiety.

This was later supported by the synthesis and testing of a PALA analog where the nitrogen is replaced with an oxygen, resulting in a 60-fold decrease in affinity (Farrington et al., 1985). This was attributed to the presumed lower pH of the carbonyl oxygen of the ester versus the amide of PALA. Since the reaction mechanism of ATCase is presumed to contain a tetrahedral intermediate, it was surprising that replacement of the trigonal carbonyl moiety of PALA with a tetrahedral sulfone group resulted in a compound with markedly reduced inhibition capability relative to PALA (Farrington et al., 1985). This effect, however, may be due to the large tetrahedral sulfone moiety that could exceed the steric restraints of the carboxamidoyl region of the ATCase active site.

3.1.1 DESIGN OF RESEARCH

Previous work has determined some of the structural requirements for tight binding of ligands to the active site of ATCase. The binding preferences for each of the regions of the active site that bind the different functional moieties of both substrates should be taken into account when designing bisubstrate analogs for optimal inhibition of the enzyme. It is clear that a four-carbon dicarboxylic moiety in the cis conformation is essential for interaction with the dicarboxylate region. In addition it is well acknowledged that a phosphate group is required for interaction with the phosphate binding region.

The region that binds the amino group of aspartate is not well defined. While it could be reasoned that there is significant steric hindrance to the binding of an amine group since the binding constant for aspartate is much higher than that of succinate, results with the bisubstrate analogs suggest that the presence of a nitrogen at this position may actually be preferred. Further work is needed to define the structural features for optimal interaction with this region. The carboxamidoyl region of ATCase is of obvious importance since it is the region whereby the transcarboxylation reaction occurs. In order to probe this region, three different approaches were utilized in order to gain insight into the structural and ionic requirements of this important and catalytically relevant region.

A series of analogs of carboxamidophosphate were tested for inhibitory effects on the catalytic subunit of ATCase from E. coli. The bacterial enzyme was used as a model for the mammalian enzyme and was used in the absence of the regulatory subunits to simplify kinetic analysis. All of the compounds contained a phosphate or phosphonate moiety to interact with the phosphate region and contained additional substituents that would be expected to extend into the carboxamidoyl region.

Bisubstrate analogs of ATCase are potentially more useful inhibitors than compounds that are similar to either substrate alone. Such an analog is not only more specific in its inhibition (and thereby exhibit reduced toxicity if used in a clinical setting), it can also tap the reserve of binding energy available to both substrates. However, it is generally accepted that an enzyme realizes its maximum binding energy upon binding the transition state of the reaction and that this facilitates catalysis (Wolfenden, 1976). Thus transition state analogs should be the tightest bound inhibitors and it has been estimated that an enzyme’s affinity for the transition state of the reaction it catalyzes can be in the order of 10^12 M. The tightest binding inhibitor of ATCase known to date is PALA (Figure 11). Since this compound is simply a bisubstrate analog, it was hypothesized that a tighter binding analog could arise by positioning a negatively charged tetrahedral moiety in the carboxamidoyl region. Thus the following compounds were synthesized and tested for interaction with ATCase:

\[
\begin{align*}
\text{S} & \quad \text{C(O)} \quad \text{CH}_{2} \quad \text{PO}_{4}^{3-} \\
\text{S} & \quad \text{C(O)} \quad \text{CH}_{2} \quad \text{PO}_{4}^{3-}
\end{align*}
\]

The first one is the sulfur analog of PALA with trigonal geometry in the carboxamidoyl region, while the next three compounds incorporated a tetrahedral and ionic phosphate moiety designed to interact with the carboxamidoyl region. The S-5-phosphoryl and S-5-phosphoronyl compounds incorporate an additional phosphate moiety that should interact with the phosphate region of the enzyme, and thus putatively have the structural requirements for binding to all four regions of the enzyme. The relative affinities of the compounds compared with several previously reported bisubstrate analogs should yield more information on the geometric preference of the critical carboxamidoyl region. A preference for a
tetrahedral moiety would lend some support to the possibility that ATPase promotes the reaction by geometric desensitization of the substrates.

The last approach to studying ATPase was the synthesis of two affinity reagents, both analogs of the reversibly inhibitor phosphoanacetic acid, that were designed to modify nucleophilic groups in the carbamoyl region of the active site. Protection studies with reversible inhibitors that are analogs of one or both substrates were done to outline the region of the enzyme modified by the reagent. These two affinity reagents could then be used to define the mode of binding of new inhibitors of the enzyme. A small reactive analog of carbamoyl phosphate would not be specific enough to inactivate only ATPase within a tumor cell, but an efficient inactivator of this type may be used to design a promising bisubstrate analog inactivator by incorporation of a dicarboxylate moiety into the inhibitor structure, resulting in a high degree of specificity for ATPase. An irreversible reagent of this type could be therapeutically useful only if it were stable enough to avoid reaction in the cell prior to combining with the target ATPase. The metabolic block produced by the reversible inhibitor PAPA can be overcome by an accumulation of intracellular carbamoyl phosphate concentrations, which may explain some of the disappointing clinical results seen with PAPA (Gualla et al., 1980). In contrast inactivation of ATPase would produce a permanent metabolic block until new enzyme is synthesized to replace it.

A suicide inhibitor of ATPase would be the optimal compound for reducing tumor cell ATPase activity. One of the compounds in this report, 6-phosphoanacetoyl-aspartate had the potential to inactivate ATPase during the catalytic mechanism. It is possible that nucleophilic groups in the carbamoyl region of the enzyme could attack the thiocarbonyl bond and thereby result in scission of the enzyme. Therefore the thioester was studied for both reversible and irreversible inhibition of ATPase.

It is hoped that the different methods of investigating the carbamoyl region of the enzyme will help to elucidate the structural and anionic requirements for optimal interaction with this region and thereby contribute to the knowledge required to design new and clinically effective inhibitors of this target enzyme.

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3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

\[^{14}C\]-Carbamoyl phosphate was purchased from New England Nuclear and was diluted with unlabeled dilithium carbamoyl phosphate in 200 mM sodium formate buffer (pH 3.8) and stored at 50 mM concentration (3.1 mCi/mol) at -70° C. Bromocresol green, phosphorus oxychloride and triethylamine were from E.M. Chemical Co. D.L-mercaptoethanol, methylthiophosphonic acid, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), malachite green osazone and thionyl chloride were from Aldrich and acetonitrile was from Fisher Scientific. 3-Phosphonopropionic acid was from Fluka. Dichloroacetyl phosphonate, carbonyl di phosphate, and peryodiphosphonate were gifts from Dr. D.W. Hutcheson (O. of Warwick, England). Hydroxymethylphosphonate and hypophosphonate were gifts from Dr. B. Oberg (Medivir, Sweden). Dilithium carbamoyl phosphate, L-aspartate, nicotinamide adenine dinucleotide (reduced form, NADH), dihydrorubinate, sodium carbonate, caesodylic acid, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonate (HEPES), pyrophosphoryl tetrachloride, pyridine, lithium chloride 1-amino-2-naphthol-4-sulfonic acid, sodium sulfate and sodium bisulfite were from Sigma Chemical Co. Glatinate-oxaloacetate transaminase and aslulate dehydrogenase were from Boehringer-Mannheim.

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2.2.2 ENZYME ISOLATION

Aspartate transcarbamoylase was isolated from E. coli using the method of Garbett and Holubek (1967) by Duncan K.K. Chang, and was stored in 40 mM potassium phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. The catalytic subunits (a) were separated from the regulatory subunits (b) by reaction with neohydroxine followed by SEPA- sephadex chromatography (Schonaa, 1972). The purified catalytic subunits were stored in 40 mM potassium phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA.

The protein concentration of the purified catalytic subunit was determined from absorbance at 280 nm, on the basis that the absorbance of a 1 mg/ml solution (1 cm path length) of the catalytic subunit is 0.72.

3.2.3 \[^{14}C\]-CARBAMOYL PHOSPHATE ENZYME ASSAY

The method of Dwyer et al (1974) was used for measuring ATPase activity in the presence of inhibitors. Each assay contained 100 mM sodium HEPES (pH 7.5), 15 mM sodium aspartate, bovine serum albumin (50 mg/ml), 15 mM sodium aspartate and aspartate transcarbamoylase catalytic subunit (1 to 4 mg/ml) 1 inhibitor. The assay mixture was preincubated in Beckman mini Poly-O vials at 25°C for 10 min, and then the reaction was initiated by the addition of \[^{14}C\]-
carboxymyl phosphate (0°C) to bring the final volume of the reaction to 400 μl. After 15 minutes, the reaction was stopped by the addition of 100 μl of 50% trichloroacetic acid. The vials were then put in a boiling water bath for 5 min, after which the removal of 14C-carbon dioxide was completed by adding 100 μl of 0.1 M sodium carbonate to each vial. After the addition of Beckman Ready-Safe scintillation fluid, the vials were then counted on a Beckman LS-230 Liquid Scintillation Counter. In order to maintain the level of carboxymyl phosphate consumption at approximately the same level for all samples (5-25%), the amount of the enzyme in each assay was varied.

3.2.4 DATA ANALYSIS

Double reciprocal plots (Lineweaver & Burk, 1934) were constructed in order to determine the pattern of inhibition for most compounds, while the inhibition constant was estimated according to Dixon (1959). In each case, four different carboxymyl phosphate concentrations were used with four different inhibitor concentrations. Two complete K values were determined for each inhibitor tested.

3.2.5 ANALYTICAL PROCEDURES

3.2.5 (a) THIOL AND THIOL ETHER DETERMINATION

Quantitative determination of the thiol and thiol ester content of the various compounds synthesized was performed according to the method of Stadman (Stadman, 1957). Free sulfhydryl groups were quantified by a spectrophotometric assay where sample aliquots were added to 1 ml p-chloromercuribenzoate in sodium acetate buffer (0.33 M, pH 4.5) and the absorbance at 295 nm was measured. Thiol esters were detected by reaction of sample aliquots (0.1-1 ml) with 0.1 N of hydrolytl units (0.1 M, pH 9.0) for 30 min, and measuring the absorbance of the ferric hydroxamate complex at 540 nm upon addition of 1 ml of 0.1 N ferric chloride in 0.1 N HCl.

3.2.5 (b) PHOSPHATE AND PYROPHOSPHATE ANALYSIS

Quantitative phosphate group analysis was performed one of two ways for each phosphorus containing inhibitor synthesized. For the phosphate derivative the method used was that of Chelvaryan and Rudnick (1970) with the modification of Duck-Chong (1979). The compound was first digested with 70% perchloric acid at 100°C for 0.5h. Malachite green assay solution (0.8 ml) was added to the sample (1-15 ml) in 0.4 ml and absorbance at 650 nm was measured after 10 min. The assay solution was made by mixing 100 ml of solution A with 300 ml of solution B for 30 minutes, followed by filtration of the mixture. Solution A was a 4.2% solution of ammonium molybdate in 5% hydrochloric acid, while solution B was a 0.2% solution of malachite green in water.

Pyrophosphate and phosphate content was determined by the method of Puttmann and Yasada (1975). Pyrophosphate analysis was performed by adding 100 μl of molybdate reagent to 1-20 μl of sample. This was followed by the addition of 100 μl of 0.5 M 1-peracetyl ethanol and 40 μl of bisulfite solution and distilled water to make a final volume of 1 ml. Absorbance was read after 15 min at 540 nm. Phosphate analysis was performed as above but with the thiol reagent omitted. Pyrophosphate did not form a significant amount of colored complex in the absence of thiol. Molybdate reagent was 2.5% ammonium molybdate in 5 N H2SO4. Bisulfite solution contained 20 mM sodium sulfite, 10 mM 1-amin-2-naphthol-4-sulfonic acid and 0.77 M sodium bisulfite.

3.2.5 (c) ASPARTATE ANALYSIS

Aspartate content was determined by an enzymatic assay which coupled free aspartate concentration to NADH oxidation (Poole and Lipscomb, 1981). The assay mixture contained sodium HEPES (100 mM), o-ketoglutarate (2 mM), NADH (1.5 mM), BSA (50 μg/ml), glutamate-oxaloacetate transaminase (50 μg/ml) and malate dehydrogenase (2.4 μg/ml) in a final volume of 0.8 ml. Initial absorbance was read at 340 nm and the rapid drop in absorbance changes upon the addition of sample or known amounts of aspartate standard were measured.

3.2.6 CHEMICAL SYNTHESIS

All of the starting materials and solvents were reagent grade or higher in purity and used without further purification.

3.2.6 (a) PHOSPHONOACETYL CHLORIDE

Phosphonoacetyl chloride was prepared according to the procedure of Skyrzyt et al (1974). Phosphonoacetic acid (0.2 g, 1.4 millimoles) was suspended in 5 ml of thionyl chloride and protected from moisture with a calcium chloride drying tube. The mixture was stirred for 24 h and maintained at 50°C with an oil bath. Excess thionyl chloride was removed by rotoevaporation, and then addition and rotoevaporation of acetonitrile. For enzyme inactivation studies, the acyl chloride was dissolved at a concentration of 2 or 1 mM in acetonitrile and added in very small aliquots to the ATCase in ossoylate buffer (pH 7.0), while the control had the same volume of acetonitrile added. After the two minutes incubation period, an aliquot was removed and diluted 25-fold into enzyme dilution buffer on ice to stop the inactivation. An aliquot of this mixture was then assayed for remaining ATCase activity.
For the protection studies with various ligands, the enzyme was preincubated with or without ligand for ten minutes prior to the addition of phosphonoacetyl chloride. The label was added as described above and the remaining ATCase activity was determined.

3.2.6 (b) PHOSPHONOPEROXICATE

Synthesis of phosphonoacetate and assay for paraacid was according to the method of Phillips et al. (1973) for other organic peracids. Phosphonoacetic acid (0.2 g, 1.4 mmol) was dissolved in 308 hydrogen peroxide with 1% sulfuric acid as a catalyst and kept at 25°C for 48 h. The hydrogen peroxide was removed with rotovaporation, and an aliquot of the remaining paraacid was diluted with 450 ml of 4 M sulfuric acid at 0°C, and then rapidly titrated to a pink end point with 0.100 M potassium permanganate to check for residual hydrogen peroxide. Phosphonoacetic acid was determined by the addition of 2 ml of saturated aqueous sodium iodide to the same solution and rapid titration of the paraacid-liberated iodide ion with 0.1 M sodium thiosulfate. For the inactivation studies, aliquots of the paraacid in cacodylate buffer (50 mM, pH 7.0) were added to the enzyme in the same buffer. After the incubation period of four minutes, aliquots were removed and diluted 250-fold into enzyme dilution buffer. Aliquots of this solution were then assayed for remaining ATCase activity. For the protection studies, the enzyme was preincubated with or without ligands for 10 minutes prior to the addition of phosphonoacetate.

3.2.6 (c) S-PHOSPHONOCETYL-MERCAPTOACETIC ACID.

Phosphonoacetyl chloride (0.20 mmol) was diluted with 2 ml of anhydrous acetonitrile. Mercaptoacetic acid (0.030 g, 0.20 mmol) was dissolved in dry acetonitrile (1 ml) with triethylamine (0.011 g, 0.06 mmol). The phosphonoacetyl chloride solution was added dropwise to the mercaptoacetic mixture on ice over a period of 5 min. After a reaction period of 1 h with stirring at 22°C, the reaction mixture was diluted in water, the pH was adjusted to 7.0 with 0.1 M HCl and the mixture was then loaded on a polyethyleneimine cellulose column (2.0 x 25 cm). The column was eluted with a linear gradient of 0.1 to 1.0 M lithium chloride. Fractions were tested for free sulphydryl groups and thiol content. The thiol ester positive fractions were pooled and desalted with Dowex-50 (H⁺ form). After rotovaporation to dryness, the samples were analyzed for thiocysteine content by the hydroxylamine assay and for phosphate content after perchloric acid digestion. In addition, free thiol content was determined before and after acid hydrolysis of the thiocysteine. Samples that showed >95% pure thiocysteine by weight were used for enzyme studies.

In order to investigate the possibility that this analog was hydrolyzed by the enzyme, an aliquot of S-phosphonoacetyl-(2.1)-mercaptoacetic was incubated with or without enzyme at 37°C for a period of 1 to 4 hours, and the level of free thiol present in the mixture was determined. Since the thiol ester had the potential to inactivate the enzyme by acylation of a nucleophilic group in the active site, the possibility of thiol ester inhibition of the enzyme was investigated. Enzyme was incubated in the presence or absence of inhibitor at a concentration two times its Kᵣ value for 1 to 6 hours at 37°C. The enzyme-inhibitor mixture was diluted 100-fold into an activity assay and ATCase activity was determined.

2.6 (d) S-PHOSPHONYL-MERCAPTOACETIC ACID.

Mercaptoacetic acid (0.030 g, 0.2 mmol) was dissolved in dry acetonitrile and triethylamine (0.10g, 1.0 mmol). Phosphorus oxychloride (0.25 mmol) was diluted with 1 ml of dry acetonitrile and added dropwise to the mercaptoacetic acid mixture over a 10 min period. After 1h the products were filtered to remove triethylamine hydrochloride and solvent was removed with rotovaporation. The residue was dissolved in water, the pH was adjusted to 7.0 with HCl (0.1 M) and the solution was loaded on a Dowex-1 column (2.9 x 25 cm, chloride form). The column was eluted with a linear gradient of 0.1 M to 1.0 M lithium chloride. Fractions aliquots were assayed for free sulphydryl groups before and after 0.1 M sodium hydroxide hydrolysis by the p-chloromercuribenzoate method. In addition, free phosphate content before and after perchloric acid hydrolysis was also determined. Fractions that contained free thiol groups after hydrolysis were pooled, and loaded on a Dowex-50 column (H⁺ form). To the eluent was added three volumes of ethanol and after 24h the precipitate was collected by filtration, washed rapidly with cold ethanol and ether and stored in a desiccator. The purity of the samples obtained were determined by quantitative analysis of the thiol and phosphate groups generated upon hydrolysis and by polyethyleneimine cellulose TLC. Samples which showed less than 5% contamination from hydrolysis products were used for enzyme assays.

3.2.6 (e) S-PHOSPHONYL-MERCAPTOACETIC ACID.

Mercaptoacetic acid (0.030 g, 0.2 mmol) was dissolved in a mixture of dry acetonitrile and triethylamine (0.14 g, 1.4 mmol). Phosphonyl tetrachloride (0.050g, 0.2 mmol) was mixed with to 1 ml of dry acetonitrile and added dropwise to the mercaptoacetic acid mixture over a 10 min period, and then the mixture was stirred for 1h. The products were filtered to remove the triethylamine hydrochloride and the solvent was removed with
rotoevaporation. The residue was dissolved in distilled water, the pH was adjusted to 7.0 with 10 mM HCl and then the sample was loaded onto a Dowex-1 column (2.0 x 25 cm, Cl\text{"} form). The column was eluted with a linear gradient of 0.1 M to 1.0 M lithium chloride. Fraction aliquots were assayed for free sulfhydryl groups before and after hydrolysis with 0.1 M NaOH. The pyrophosphate and phosphate content of the same fractions was also determined before and after hydrolysis. When the fractions were assayed for pyrophosphate prior to base hydrolysis, it was found that a slow time-dependent release of pyrophosphate occurred, presumably due to the hydrolysis of S-pyrophosphoryl-

The molybdate/sulfuric acid reagent. This release was followed over time which allowed extrapolation back to zero time to calculate the amount of free pyrophosphate which was present in the fraction. Fractions that did not contain any detectable free thiol groups, phosphate or pyrophosphate before base hydrolysis but that generated stoichiometric amounts of free thiol and pyrophosphate upon hydrolysis were combined and run through a Dowex-50 column (H\text{"} form). To the eluent was added three volumes of ethanol. After 24 hours at 4°C, the precipitate was collected by filtration, washed rapidly with cold ethanol and then ether and stored promptly in a vacuum desiccator to minimise hydrolysis which occurred with
time. The purity of the samples obtained were determined by quantitative analysis of the thiol and pyrophosphate groups generated upon hydrolysis and by polyethyleneimine cellulose TLC. Samples which showed less than 3% contamination from hydrolysis products were used immediately for enzyme assays.

3.2.6 (1) N-PYROPHOSPHORYL-L-ASPARTATE

N-pyrophosphoryl-L-aspartate was prepared under conditions previously used for the synthesis of N-phosphoryl-

Ampicillin (0.167 g, 1.10 mmol) was dissolved in water, the pH was adjusted to 8.0 with 1M NaOH and magnesium oxide (0.33 g, 8.25 mmol) was added. Pyrophosphoryl tetrachloride (0.28 ml, 1.33 mmol) was diluted with 2.0 ml of carbon tetrachloride and added dropwise over a period of 10 min to the aspartate solution with vigorous stirring. The reaction was kept on ice and stirred for an additional 2h. The pH remained in the vicinity of 8 during the reaction. The mixture was filtered with suction, and the precipitate which consisted of excess magnesium oxide and magnesium pyrophosphate was discarded. After separating and discarding the organic phase the clear filtrate was adjusted to pH 7.0 with dilute acetic acid. The solution was chilled and then 2 volumes of cold ethanol were

added slowly with stirring. The resulting precipitate was collected with suction filtration. Magnesium chloride and unreacted aspartate remained in the filtrate. The precipitate was washed with absolute ethanol and then ether. The crude magnesium salt of pyrophosphoryl-L-aspartate was dissolved in water and treated with 2 volumes of cold ethanol. The precipitate was collected, washed with ethanol and ether and dried several hours in a vacuum desiccator.

Pyrophosphate and aspartate content was determined before and after treatment with acetic acid (0.1 M) to remove the pyrophosphate group from the aspartyl nitrogen. Samples with less than 5% contamination from the hydrolysis products (free aspartate and pyrophosphate) were used immediately for enzyme assays. Since the enzymic aspartate assay required the amino group of this amino acid to be free in order to detect it, and there was an equimolar release of aspartate and pyrophosphate after treatment with acetic acid, this indicated that the product was indeed N-pyrophosphoryl-L-aspartate. The stability of N-pyrophosphoryl-L-aspartate at pH 7.5 was determined by measuring the increase in free aspartate concentration over a 12h period using the enzyme coupled aspartate assay. 31P-NMR in H2O yielded two doublets, one at -1.04 ppm and one at -10.65 ppm with J = 18.5 Hz. For comparison, creatine phosphate (with an -NH-P=O group) showed a peak at -2.4 ppm, while pyrophosphate yielded a peak

showed a peak at -2.4 ppm, while pyrophosphate yielded a peak at -6.2 ppm.
3.3 RESULTS

3.3.1 CARBAMYL PHOSPHATE ANALOGS

The carbamyl region of the enzyme was investigated by testing the inhibitory effects of compounds with a phosphate or phosphonate moiety that had varying side chains capable of extending into the carbamyl region. Since all compounds inhibited the catalytic subunit of ATCase in a competitive manner, it was presumed that the phosphate group of each inhibitor (or one of the phosphate groups in the compounds with two) interacted with the phosphate binding region and the rest of the molecule interacted with the carbamyl region. However, these results do not rule out the possibility that the remaining portion of the inhibitor interacted with the dihydroxyacetone region of the enzyme.

The tight competitive inhibition of ATCase previously reported for pyrophosphate (Porter et al., 1969) and phosphonofluoride (Dennis et al., 1986) was interpreted as a preference for a negatively charged moiety in the carbamyl region of the active site. This is consistent with the proposed catalytic mechanism whereby a negative charge develops on the carbonyl carbon of carbamyl phosphate to form a tetrahedral intermediate. Therefore, the structure-activity relationships of stable carbamyl phosphate analogs which maximize interactions with the carbamyl region are important in the design of potent bisubstrate analogs or transition state analogs.

The inhibitory effects of several phosphate or phosphonate containing compounds are presented in Table XIV. It is clear that the distance between the negative charges on the two phosphorus atoms has a significant effect on affinity for ATCase. When the bridging oxygen of pyrophosphate is removed to form hypophosphate, the compound binds about three times tighter than pyrophosphate, whereas insertion of an extra bridging oxygen as in pyrophosphate results in a four-fold decrease in affinity. It is significant that hypophosphosphate and phosphonofluoride have the same affinity for ATCase. This suggests that the tetrahedral phosphate group is not preferred over the trigonal carbonyl group for binding to the carbamyl region of the active site.

The importance of the nature of the bridging group of pyrophosphate was further examined with various diphenyl phosphate compounds. It was previously established that substitution with a methylene group has little effect on the potency of inhibition observed (Dennis et al., 1986). Substitution with an amino group caused a slight decrease in affinity compared with pyrophosphate, while replacement with a dichloromethylene group resulted in a slightly increased potency of inhibition. An hydroxymethylmethylene group placed in between two phosphate groups resulted in a 6- to 10-fold loss in affinity relative to pyrophosphate and methylene diphenyl phosphate respectively. This is probably due to steric hindrance created by the binding of the methyl group. In contrast, when the bridging oxygen of pyrophosphate is replaced by a carbonyl group, a potent carbamyl phosphate analog inhibitor is formed.

The Dixon plot for carbonyl diphenyl phosphate is presented in Figure 12. It had a remarkably low binding constant of 3.5 μM, binding 6- to 10-fold tighter than phosphonofluoride or methylene diphenyl phosphate, the analogs with the highest affinity for ATCase yet reported (Dennis et al., 1986). The difference in affinity between the methylene and carbonyl compounds may be due to positive interaction of the carbonyl group with the carbamyl region. However, it is just as likely that the increased affinity for carbonyl diphenyl phosphate relative to methylene diphenyl phosphate or pyrophosphate is due to the effects of the trigonal carbonyl group which alters the relative position of the two phosphate groups. This structural difference may allow maximal interaction of the second phosphate group with the positively charged residues within the carbamyl region. Since these results underlined the importance of the adjacent carbonyl group of carbonyldiphenyl phosphate for effective inhibition, some additional compounds with a phosphate moiety and a carbonyl or carboxylate moiety were tested for...
Figure 12: Dixon Plot for the Inhibition of ATCase by Carbonyl diphosphonate. ATCase was preincubated with various concentrations of inhibitor for 10 minutes prior to the addition of various concentrations of \(^{14}C\)-carbonyl phosphate to initiate the reaction. After 15 minutes the reaction was stopped with trichloroacetic acid and treated as described in the Methods section.

Inhibition.

Early studies on ATCase indicated that phosphoenolpyruvate had a moderate affinity for the enzyme (Porter et al., 1969), while its shorter homologue PFA was later reported to be a seven-fold better inhibitor (Dennis et al., 1986). The present work found phosphoenolpyruvate acid to be an extremely weak inhibitor (Table XV). As with the diposphosphate or diphasphonate compounds in Table XIV, it is apparent that increasing the distance between the carboxylate and phosphate groups of PFA causes a loss in affinity for the enzyme (Table XV). The large inhibition constant for phosphoenolpyruvate acid suggests that it exceeds the steric constraints of the carbamoyl phosphate regions of the active site. Similarly when one of the carbonyl oxygens of PFA is replaced with an ethylamido group, the compound exhibits a weak affinity for the enzyme. The relative large and hydrophobic ethyl group was not expected to interact in a positive manner with the polar carbamoyl region of the active site and would most likely reduce the interactions of the adjacent carbonyl moiety with this region.

The importance of interactions with the free oxygens of PFA is underlined by the fact that methylation of one of these oxygens causes a five-fold loss in affinity relative to PFA.

### Table XV

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( K_i ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p\text{-}p\text{-CO}_2^{-} ) (PFA)</td>
<td>22 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>( p\text{-}p\text{-}CH_2\text{-CO}_2^{-} )</td>
<td>150 ± 20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>( p\text{-}p\text{-CH_2\text{-}CH_2\text{-CO}_2^{-}})</td>
<td>7500 ± 320</td>
</tr>
<tr>
<td>2-( p\text{-}p\text{-}CH_2\text{-CH_2\text{-CO}_2^{-}})</td>
<td>2000 ± 180</td>
</tr>
<tr>
<td>( p\text{-}p\text{-CH_3\text{-CO}_2^{-}})</td>
<td>128 ± 24</td>
</tr>
</tbody>
</table>

<sup>a</sup> Dennis et al., 1966 <sup>b</sup> Porter et al., 1969.
3.3.2 DICARBOXYLATE DERIVATIVES

The inhibitory effects of some newly synthesised bisubstrate or transition state analogs on ATCase were examined. Each inhibitor contained the four-carbon dicarboxylate moiety found in aspartate, with different α-substituents. Each compound exhibited competitive inhibition versus carbamoyl phosphate and therefore interacts with the regions of the enzyme associated with binding this substrate. While the dicarboxylate moiety of each inhibitor is presumed to interact with the dicarboxylate region of the enzyme, the possibility of an alternative mode of interaction at the active site cannot be ruled out. The following discussion is therefore based on the presumption that a normal mode of binding is exhibited by each inhibitor.

An analog of PALA with the amid nitrogen replaced by a sulfur atom exhibited an inhibition constant similar to that previously reported for the oxygen analog of PALA (Table XVI) (Farrington et al., 1985). Although the sample used for enzyme assays was at least 97% pure thioester by weight, it is possible that a small amount of a potent contaminant is responsible for the inhibition seen. However, the most common contaminants seen in the samples were the components of the thioester, namely phosphonoacetate and aspartogluconate and these compounds have IC₅₀ values for ATCase that are 50- and >100-fold larger than the thioester sample respectively, it appears to be unlikely that a contaminant is responsible for the inhibition seen. To rule out the possibility that the sulfur analog was hydrolyzed by the enzyme, an aliquot of 6-phosphonoacetyl-(2-L)-aspartogluconate was incubated without or with enzyme at 37°C for a period of 1 to 6 hours, and the level of free thiol present in the mixture was determined. No hydrolysis of the inhibitor occurred during the incubation period. Since the thiol ester had the potential to inactivate the enzyme by covalent modification of a nucleophilic group in the active site, the possibility of time-dependent inhibition of the enzyme was investigated. Enzyme was incubated in the presence of inhibitor at a concentration two times its IC₅₀ value for 1 to 6 hours at 37°C. After dilution of the enzyme-inhibitor mixture 100-fold into an activity assay, there was no significant difference in activity compared to enzyme incubated in the absence of inhibitor. Thus the compound was found not to inactivate the enzyme and is therefore a reversible and stable inhibitor of ATCase. Under the presumption that only the L isomer of the D,L mixture is able to inhibit the enzyme, the IC₅₀ value for this isomer would be approximately 2.75 μM, one hundred times greater than that of PALA.

Those results indicated that the nitrogen of PALA makes significant interactions with the enzyme and contributes to the binding affinity of bisubstrate analogs.

<table>
<thead>
<tr>
<th>Inhibitor (R = -CH₂ – CO₂⁻)</th>
<th>Kᵢ⁻ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O–P–(CH₂–CO₂⁻)</td>
<td>0.027⁹</td>
</tr>
<tr>
<td>O–P–CH₂–C–NH–R (L)</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>O–P–CH₂–C–S–R (DL)</td>
<td>2.0⁶</td>
</tr>
<tr>
<td>O–P–CH₂–C–O–R</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>O–P–CH₂–C–NH–R</td>
<td>50 ± 8⁴</td>
</tr>
</tbody>
</table>

* The nature of inhibition was determined to be competitive against carbamoyl phosphate for all compounds tested in this work.
* Data from Collins & Stark, 1971.
* Data from Farrington et al., 1985.
* Data from Dennis et al., 1986.
Since these compounds simply combine the features of both substrates into one molecule, it was reasoned that the enzyme could bind an analog of the transition state much more tightly. For this reason, an aspartate analog that incorporated a tetrahedral phosphate moiety poised to interact with the carbamyl region where the proposed tetrahedral transition state develops was synthesized. In addition it had a second phosphate moiety that would extend into the phosphate binding region as does the phosphate moiety of PALA. The affinity of the putative transition state analog for NCCase was significantly weaker than expected (Table XVI). It bound 5-fold less tightly to the enzyme than the bisubstrate analogue PALA.

It should be noted that the two major contaminants in the N-pyrophosphoryl-L-aspartate preparation were found to be aspartate and pyrophosphate. Since aspartate is the substrate for the enzyme and pyrophosphate has an IC₅₀ value that is two hundred-fold larger than that exhibited by the N-pyrophosphoryl-L-aspartate preparation (see Section 3.3.1), the inhibition observed is therefore most likely due to the aspartyl derivative and not a contaminant.

The importance of the second phosphate group for binding was determined by comparing the affinity of the pyrophosphoryl derivative with the previously synthesized inhibitor N-phosphoryl-L-aspartate (Dennis et al., 1986).

N-phosphoryl-aspartate. Not surprisingly, addition of a second phosphoryl group to form S-phosphoryl-L-mercaptosuccinate resulted in an increase in potency of inhibition. However, the 7-fold enhancement of binding observed with the S-derivatives was significantly smaller than the 200-fold enhancement observed by the addition of the second phosphoryl group to N-phosphoryl-L-aspartate. This implies that not only does the NH group of aspartate significantly contribute to the overall binding affinity observed with bisubstrate/transition state analogs, it appears to influence the level of interactions that occur in the adjacent regions.

3.3.3 INACTIVATION WITH AFFINITY ANALOGS

Two affinity analogs of the inhibitor phosphonoacetate were synthesized with the goal of using these compounds as probes to investigate the mode of binding of several of the inhibitors tested in this work. The first affinity label was phosphonoacetyl chloride, and initial inactivation studies done found that significant inactivation occurred upon incubation of NCCase with 2.0 mM of the label and a two minute incubation period. In order to determine the selectivity of inactivation, protection studies were done with phosphonoacetate. Thus the enzyme was preincubated with phosphonoacetate prior to the addition of phosphonoacetyl chloride. No significant protection from inactivation was observed in the presence of phosphonoacetate at a concentration equal to double its IC₅₀ value. Since this acyl chloride was indiscriminately reactive and did not interact with the phosphate and carbamyl regions of the enzyme before inactivation occurred, it could not be used as a probe of the carbamyl region of the enzyme.

In contrast, phosphonoacetate produced a time- and concentration-dependent inactivation of NCCase. Significant inactivation was observed at 50 μM concentration with an incubation period of 6 minutes. The effect of several reversible inhibitors on the inactivation was investigated to see if a specific region of the active site was modified by this affinity reagent. Both pyrophosphate and PALA afforded protection from inactivation when present at concentrations equal to their IC₅₀ values, suggesting that these molecules obstruct the approach of phosphonoacetate to their specific target functional groups on the enzyme (Figure 13). The initial results obtained with this reagent were encouraging and suggested that the reagent interacted with the phosphate and carbamyl regions of the enzyme and the active oxygen of phosphonoacetate modified a nearby residue in the carbamyl phosphate binding region, causing inactivation. Therefore the inhibitor seemed like a useful probe of the carbamyl region and may have been used to help
determine the mode of binding of reversible inhibitors through protection studies. However, the inactivation of ATCase produced by phosphonoperacacetate proved to be highly variable among different inactivation assays so the compound was not pursued further.

3.4 DISCUSSION

The main goal of the research on ATCase was to explore and further elucidate the structural features necessary for binding to the active site. This lead to the development and testing of new structural analogs of the substrate carboxyl phosphate or of both substrates. The well-known and tight-binding ATCase inhibitor PALA resembles both substrates and there is therefore a large entropic contribution to the strength of binding of PALA. Since PALA contains a trigonal carboxyl moiety and the reaction is thought to involve a tetrahedral intermediate, it was reasoned that a tighter binding inhibitor could be synthesized by placing a charged tetrahedral moiety in the carboxyl region of a compound which otherwise resembles both substrates. Such a compound was synthesized and was found to bind less tightly to ATCase than PALA. Other inhibitors with uncharged tetrahedral groups expected to interact with the carboxyl region of the enzyme have also been reported as significantly weaker inhibitors compared with PALA. For example, compounds with a -CH\_2-CH\_2(OH)\_2 or an -NS-S(O\_2)\_2 moiety replacing the -OH-C(O\_2)\_2 of PALA are both relatively poor inhibitors of ATCase despite the presence of oxygen-containing tetrahedral groups in the proper position (Roberts et al., 1976; Farrington et al., 1985). There are several
possible factors which could contribute to the low affinity of the putative transition state analogs reported in the past and in this work.

3.4.1 ABNORMAL MADS OF BINDING

All of the bisubstrate/transition state analogs synthesized in this work and the tetrahedral inhibitors mentioned above contain a four carbon dicarboxylic acid moiety which is presumed to interact with the dicarboxylate region of the enzyme. They all have an \( \text{S} \)-substituent designed to extend into the carbamoyl region which is linked to a phosphate or phosphonate moiety to extend into the phosphate binding region. Since the compounds in the present study were only tested for their competitiveness toward carbamoyl phosphate, it is only known that they interact with the regions of the enzyme responsible for binding this substrate. There is no indication of what specific portion of the inhibitor interacts with the dicarboxylate region or if there is any interaction with this region at all. While the dicarboxylate portion of the inhibitor is fully expected to interact with the same region of the active site, the possibility that one or both of the carbonyl groups interacts with the previously established anionic binding centres within the carbamoyl and phosphate binding regions of the enzyme.

\[
\text{AvCase REACTION} \quad \text{INHIBITOR}
\]

\[
\begin{align*}
&\text{B}^+ \quad \text{BH} \quad \text{KH}^+ \\
&\text{N-} \quad \text{N-} \quad \text{N-}
\end{align*}
\]

Since the pyrophosphoryl group is attached to the nitrogen of aspartate it is forced to interact with this region. In contrast when pyrophosphate itself binds to the active site it may bind in a manner so that it minimizes interaction with the general base in the amino region of the active site. By attaching the anionic ligand to aspartate, it is possibly forced to bind in a slightly different mode than it would by itself. While one of the phosphoryl oxygens of \( \text{N-pyrophosphoryl-L-aspartate} \) would be expected to interact in a positive manner with the carbamoyl region where the transition state develops, the second phosphoryl oxygen may develop negative interactions with a residue which is poised to deprotonate the aspartate substrate. The absence of a negatively charged oxygen that could develop similar negative interactions with this residue in the structure of PALA may explain some of the greater affinity of PALA versus \( \text{N-pyrophosphoryl-L-aspartate} \).

3.4.3 GEOMETRIC VS ELECTROSTATIC DESTABILIZATION

An early model for the catalytic mechanism of AvCase suggested that geometric destabilization of the substrates relative to the transition state provided such the necessary force to promote catalysis (Jacobson & Stark, 1973). The present results suggest that tetrahedral geometry is not preferred over trigonal geometry in the position where catalysis occurs. This would suggest that geometric destabilization of the trigonal group of carbamoyl phosphate is not a large factor in promoting the reaction, or that the geometry of the transition state actually lies in between a trigonal and tetrahedral conformation.

Alternatively, the enzyme may rely more upon electrostatic destabilization of the carbonyl carbon. This is supported by crystallographic studies of AvCase complexed with PALA. Three residues interact specifically with the carbonyl carbon of the inhibitor (Kramse et al., 1987). Site-directed mutagenesis studies indicate that these three residues probably act in a concerted fashion to polarize the carbonyl group of the substrate carbonyl phosphate to make it more susceptible to nucleophilic attack by aspartate (see sections 3.1.8 and 3.1.9 (a)).

3.4.4 CONCLUSION AND FUTURE WORK

There are several different explanations for the low affinity of the newly synthesized putative transition state analogs for AvCase relative to the bisubstrate analogs.
PADA. One final explanation that cannot be completely ruled out is that the reaction does not involve a tetrahedral transition state and that it actually proceeds in a concerted manner. Thus the departure of phosphate would occur at the same time as the nucleophilic attack on the amine group. There is at present, no experimental support for such a mechanism.

One of the most significant contributions of this work is the finding of the most potent anionic inhibitor of ATPase yet reported, carbonyl diphosphonate. It would be of interest to determine the structural basis of the inhibition observed with crystallographic studies on the complex of ATPase with this anionic ligand. Finally, the development of a compound which incorporated the features of anapertate and carbonyl diphosphonate would be an area of interest for future work.

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