LIGAND INTERACTIONS AT THE ACTIVE SITE OF ASPARTATE TRANSCARBAMOYLASE FROM *ESCHERICHIA COLI*

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by

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

March, 1985.

Master of Science (1985)
(Biochemistry)

McMaster University
Hamilton, Ontario.

Title: Ligand Interactions at the Active Site of Aspartate Transcarbamoylase from Escherichia coli

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Number of Pages: xiii, 107

ABSTRACT

The carbamoyl region of the active site of aspartate transcarbamoylase from Escherichia coli was probed using an enzyme assay in which the two substrates were varied near their respective K_m's. The inhibitors tested, some synthesized and some commercially available, were chosen to satisfy the structural requirements for binding to either the dicarboxylate or phosphate region with a substituent capable of extending into the carbamoyl region. However, the dicarboxylate based inhibitors were found to bind in an abnormal manner (unlike L-aspartate or succinate on which they were based).

The carbamoyl region was found to contain a positively charged side-chain and preliminary results indicate that tetrahedral groups are not preferred over trigonal moieties. It is suggested that electrostatic stabilization of the negative charge which develops in the transition state may be a major factor in promoting catalysis. The identity of this charged group in the carbamoyl region is postulated to be His134 based on available X-ray diffraction data. The binding subsites of the active site of this enzyme were also found to be oriented in essentially the same plane. These results will greatly aid in the design of future mechanism-based

inhibitors to this enzyme that may have therapeutic value (at this time the mammalian enzyme is thought to have a similar catalytic mechanism).

ACKNOWLEDGEMENTS

I would like to thank Dr. W.W.-C. Chan for his guidance and supervision as well as my supervisory committee: Dr. T. Neilson and Dr. G.E. Gerber.

Great appreciation goes to Duncan K.K. Chong, Dr. M. Vijaya Krishna, and Dr. E. Anne Jacobson for their continuous assistance and guidance throughout this work.

Finally, I would like to thank my wife, Marilyn, for her love and support.

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ABBREVIATIONS

Å	angstrom (10^{-8} cm)
Ac	acetyl group
arg	arginine
ATCase	aspartate transcarbamoylase
ATP	adenosine triphosphate
BiBi	enzyme reaction involving two substrates and two products
c_3	catalytic subunit of aspartate transcarbamoylase from Escherichia coli
c_6r_6	aspartate transcarbamoylase
CD	circular dichroism
$CDCl_3$	deuterated chloroform
CDP	cytosine diphosphate
cpm	counts per minute
CTP	cytidine triphosphate
cys	cysteine
δ	NMR chemical shift (parts per million downfield from tetramethylsilane
DIHOP	4,5-dicarboxy-2-hydroxypentyl phosphonate
DIKEP	4,5-dicarboxy-2-ketopentyl phosphonate
D_2O	deuterium oxide
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate

his	histidine
Hz	cycles per second
IC_{50}	concentration of inhibitor reqired to cause 50% inhibition of enzyme at a specific substrate concentration
Im	imidazolium group
IR	infra-red
K_{cat}	defines the number of catalytic processes (or "turnovers") the enzyme can catalyse in unit time
K_i	inhibition constant. Equilibrium (dissociation) constant of the reaction E + I = EI
K_m	Michaelis constant. Concentration of substrate at which the velocity of the reaction catalysed by the enzyme equals one-half of V_{max}. One of the constants of the Michaelis-Menten equation.
lys	lysine
mp	melting point
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidized form/reduced form)
NMR	nuclear magnetic resonance
ORD	optical rotary dispersion
PALA	phosphonoacetyl-L-aspartate
pH	negative logarithm of hydrogen ion concentration
pHMB	parahydroxymercuribenzoate
pK_a	negative logarithm of acid dissociation constant
POPOP	p-bis[2-(5-phenyloxazolyl)]benzene
PPO	2,5-diphenyloxazole

P_i	inorganic phosphate
r_2	regulatory subunit of aspartate transcarbamoylase from Escherichia coli
r/dr	ribose/deoxyribose
R_f	ratio of distance travelled by solute to that of solvent
SDS	sodium dodecylsulphate
T	temperature (^oC)
TLC	thin layer chromatography
TMP	thymidine monophosphate
TNBS	trinitrobenzene sulfonic acid or picrylsulfonic acid
tyr	tyrosine
UDP	uridine diphosphate
UMP	uridine monophosphate
UTP	uridine triphosphate
UV	ultraviolet
V_{max}	maximum velocity: the value of v where the enzyme is saturated with substrate. One of the constants of the Michaelis-Menten equation.

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1. INTRODUCTION

A large variety of molecules, both naturally occuring as well as synthetically made, have been shown over the past decade to cause specific inhibition of some target enzymes. The outcome of this chemical intervention in the biological system can be either beneficial or detrimental. An understanding of chemical inhibition at the molecular level can help not only in the study of the nature of enzymatic catalysis but also in the rational design of drugs with maximal *in vivo* specificity.

The enzyme aspartate transcarbamoylase from *Escherichia coli* (EC 2.1.3.2) catalyses the reaction of carbamoyl phosphate with L-aspartate to form N-carbamoyl-L-aspartate and phosphate (Reichard & Hanshoff, 1956). This reaction is the first unique step in pyrimidine biosynthesis. The synthesis (Yates & Pardee, 1957) and activity (Shepherdson & Pardee, 1960) of the enzyme is regulated very tightly. These two features have led to a close scrutiny of aspartate transcarbamoylase. This enzyme has been studied both by enzymologists interested in understanding regulatory mechanisms as well as by researchers hoping that an inhibitor of this pathway can be developed as an antineoplastic agent (Swyryd *et al.*, 1974). Because of the ease with which the catalytic properties of

the E. coli aspartate transcarbamoylase can be isolated, the bacterial enzyme has been used as a model for the mechanism of the mammalian enzyme (part of a large multi-enzyme complex (Jacobson & Stark, 1973)). Thus the aim of this research is to describe further the catalytic mechanism of the E. coli aspartate transcarbamoylase in terms of the structure of the transition state.

1.1. ENZYME KINETICS

Structural and kinetic studies have shown that enzymes have well defined binding sites for their substrates, sometimes form covalent intermediates, and generally involve acidic, basic, and/or nucleophilic groups. These features are necessary for catalysis and may be utilized in the design of potent enzyme inhibitors.

1.1.1. TRANSITION STATE THEORY

Transition state theory greatly simplifies the understanding of the principles involved in catalysis and is particularly useful for analysis of structure-reactivity relationships. In this theory, the processes by which the reagents come in contact with each other are ignored. The only physical entities considered are the reagents, or ground state, and the most unstable species on the reaction pathway, the transition state. In the transition state, chemical bonds are being formed and broken. The transition

state should not be confused with intermediates. Intermediates are chemical species whose bonds are fully formed and occupy the troughs in the reaction coordinate diagram (transition states occur at the peaks). By considering that the transition state and the ground state are in thermodynamic equilibrium, the concentration of the transition state can then be calculated from the difference in their energies and thus, the rate of the reaction may be derived.

1.1.2. HAMMOND POSTULATE

The Hammond postulate is very useful for the application of transition state theory on the analysis of structure-reactivity data. This postulate states that if there is an unstable intermediate in the reaction pathway, the transition state for the reaction will resemble the structure of this intermediate (Hammond, 1955). Therefore, the enzymologist that knows the structure of such an unstable intermediate may predict the structure of the transition state.

1.1.3. RATE ACCELERATION

Chemical reactions in solution are often very slow while enzyme catalysed reactions may often proceed faster by a facter of 10^{10} or more (Wolfenden, 1976).

The bringing together of the catalysts and substrates in the correct orientation involves a large loss

of entropy. However, since the enzymic reactions take place within the confines of the enzyme-substate complex, the catalytic groups can be considered to be part of the same molecule as the substrate (giving a very high effective concentration). As a result, there is no loss of translational or rotational entropy in the transition state. This loss of randomness is counteracted by the enzyme-substrate binding energy (lowering the observed dissociation constant). Therefore, the rotational and translational entropies of the substrate are lost on formation of the enzyme-substrate complex and not during the chemical steps.

The other major method by which an enzyme may accelerate the rate of reaction is by the destabilizion of the substrate. This destabilization may be accomplished by a combination of electrostatic or geometric strain (limited because enzymes are not rigid structures) being placed upon the substrate or by desolvating the substrate (Wolfenden, 1976).

In conclusion, it is advantageous for the enzyme to apply the maximum binding energy to the transition state, not the substrate or product.

1.2. ENZYME INHIBITORS

1.2.1. REVERSIBLE INHIBITORS

The structure of the transition state of an enzymic reaction can be partially deduced from organic reaction

mechanisms. By synthesizing and measuring the enzyme's affinity for analogues which mimic the transition state, a more detailed description of the catalytic mechanism of the enzyme can be proposed. By refining the inhibitors designed upon the modified model, the description of the catalytic method can become more detailed. At this point highly potent and specific reversible inhibitors may be developed that may have the desired pharmacological activities.

1.2.2. IRREVERSIBLE INHIBITORS

Irreversible inhibitors inactivate the target enzyme by covalent derivatization of the enzyme protein or of a tightly bound cofactor. Irreversible inhibitors may be divided into two classes: 1) those compounds that are structural analogues of the normal substrate but contain a reactive group (often called affinity labels or active site-directed reagents); and 2) those compounds that contain a latent reactive functional group, activated only by catalysis by the target enzyme (often called mechanism-based inactivators, k_{cat} inhibitors or suicide inhibitors) (Walsh, 1982).

The first group of inhibitors have been used for active site structual mapping of purified enzymes. However, because the reactive functional groups are already present when the molecules are in cellular solutions before reaching the target enzyme, they are in general too indiscriminately

reactive to be of pharmacological interest.

It is the second group of inhibitors which shows the extremely high specificity desired for pharmacological agents. For the design of such compounds, the environment of the active site and catalytic mechanism of the target enzyme must be known in some detail.

1.3. ASPARTATE TRANSCARBAMOYLASE

Since it is the aim of this research to develop and test enzyme inhibitors to describe further the catalytic mechanism of the E. coli aspartate transcarbamoylase, it is appropriate to review the current knowledge concerning this enzyme.

1.3.1. OLIGOMERIC PROTEINS AND ALLOSTERIC INTERACTIONS

The activation of oligomeric enzymes, some of which catalyze reactions occurring at the branch points between various metabolic pathways, are thought to be modulated by interactions between the subunits of the protein. These interactions are caused by binding to the protein of products of the reaction or other unrelated compounds. The first enzyme in a sequence of biosynthetic reactions is often inhibited by the final product so that a large amount of unused final product will prevent its own further synthesis. This type of control is termed feedback inhibition (Umbarger, 1956).

Products of unrelated pathways can also modulate enzymatic activity by either inhibition or activation. Such effector compounds usually exert an allosteric control as they do not bind at the active site of the subunit enzyme but to an alternate site, or to a special regulatory subunit (Monod & Jacob, 1961; Monod et al., 1963).

Another form of control occurs when interactions between the polypeptide subunits cause the substrate saturation profile to become a sigmoidal curve rather than a normal hyperbola indicating a much greater activity response to small changes in substrate concentration.

When the binding of substrate molecules cause the subunit interactions, it is termed a homotropic interaction while the binding of non-substate effector molecules cause a heterotropic interaction (Monod et al., 1965).

Aspartate transcarbamoylase (ATCase) from Escherichia coli is a multisubunit enzyme which shows both homotropic and heterotropic properties. The availability of large quantities of this enzyme has allowed its detailed study. ATCase has become one of the best characterized multisubunit enzymes (for reviews see: Kantrowitz et al., 1980a&b; Jacobson & Stark, 1973; Gerhart, 1970) although a complete understanding by which the rate of reaction is modulated by the subunit interactions has not been reached.

1.3.2. ESCHERICHIA COLI ATCase--CONTROL OF PYRIMIDINE BIOSYNTHESIS

ATCase has been found to be a major regulatory enzyme in pyrimidine metabolism.

(a) CONTROL OF ENZYME ACTIVITY-FEEDBACK INHIBITION

Early in the 1950's, the addition of uracil or cytosine to the growth medium of E. coli was found to suppress the biosynthesis of pyrimidines (Bolton et al., 1952; Bolton & Reynard, 1954). Experiments in vitro and in vivo showed that cytidine, and especially cytidine-5'-phosphate, were inhibitors of carbamoyl aspartate formation (Yates & Pardee, 1956). The production of carbamoyl aspartate from aspartate and carbamoyl phosphate (Jones et al., 1955) was found to be catalysed by aspartate transcarbamoylase (Reichard & Hanshoff, 1956). This is the first unique step in pyrimidine biosynthesis (Fig. 1.1).

Purified ATCase (Shepherdson & Pardee, 1960) has been shown to be most effectively inhibited by the trinucleotide CTP (Gerhart & Pardee, 1962) but not by UTP and GTP. ATP, the product of a different pathway, is a substantial activator on this starting reaction of the pyrimidine pathway in an example of "parallel activation".

The effects of CTP and ATP on ATCase in vivo help maintain a balance between the levels of pyrimidine nucleotides and purine nucleotides as required for nucleic acid synthesis.

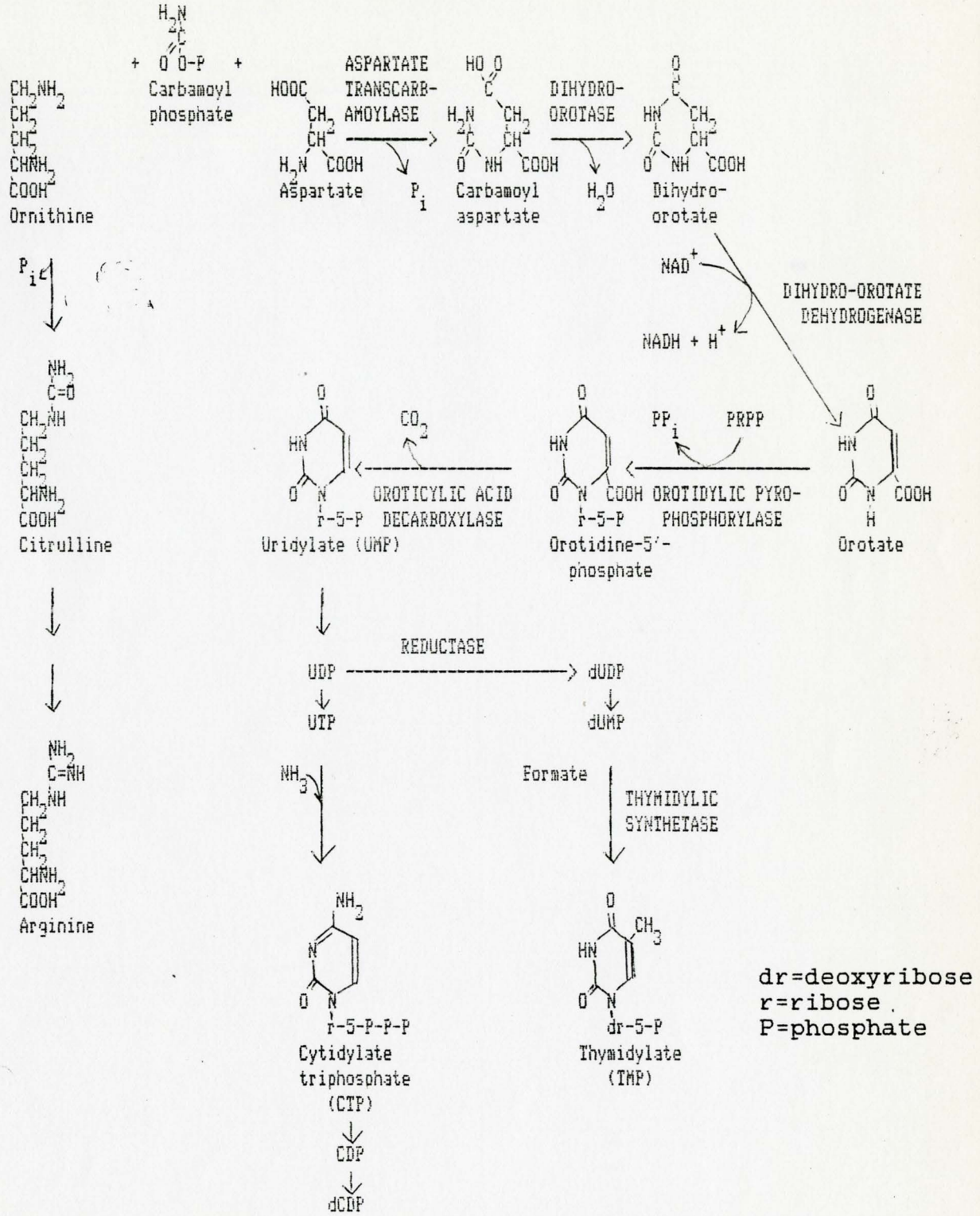


FIGURE 1.1 -- Metabolic fates of carbamoyl phosphate. (modified from Harper, 1969)

In another control point of pyrimidine biosynthesis (Fig. 1.2), UMP, one of the intermediate products of the pathway, inhibits carbamoyl phosphate synthetase. This enzyme produces starting materials for both pyrimidine and arginine biosynthesis. In arginine-free growth medium, the concentration of ornithine increases and activates carbamoyl phosphate synthetase, counteracting the effect of UMP. If arginine is supplied in the growth medium, the major regulatory enzyme of pyrimidine biosynthesis becomes carbamoyl phosphate synthetase. CTP also controls its own production by inhibiting CTP synthetase (Gerhart, 1970).

(b) CONTROL OF ENZYME LEVELS

Not only is pyrimidine biosynthesis regulated by feedback inhibition, but the amount of ATCase synthesized in the cell is controlled by a repression mechanism. The sythesis of ATCase is repressed by the addition of the negative modulator uracil to the growth medium (Yates & Pardee, 1957). This allowed the isolation of a strain of E. coli which produces extremely large quantities of ATCase (Gerhart & Holoubek, 1967); twice the normal amount of enzyme is produced under derepressed conditions because the strain is diploid in the region containing the cistrons for ATCase. This strain is also defective in the gene for orotidylate decarboxylase (Fig. 1.1) and therefore, grows slowly in the absence of

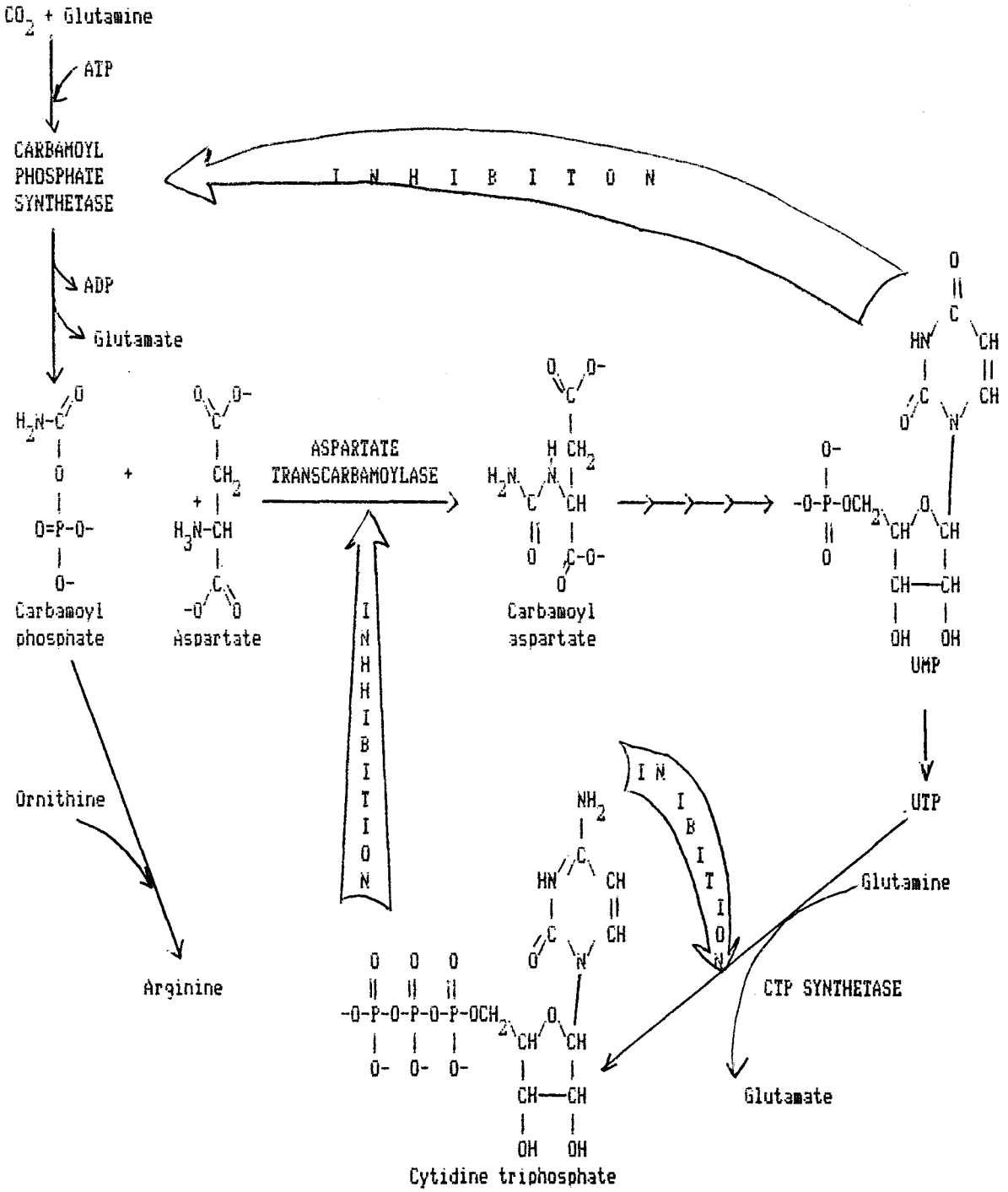


FIGURE 1.2 -- Regulatory curcuits controlling pyrimidine biosynthesis at the enzyme level in E. coli. The heavy open arrows signify the inhibitory action of a specific metabolite on one of the enzymes of the pathway (redrawn from Jacobson & Stark, 1973).

pyrimidines. To obtain large amounts of ATCase, the bacteria are first grown in the presence of uracil (repressed ATCase synthesis but rapid cell growth). When the uracil is depleted, ATCase synthesis becomes derepressed and extremely large amounts of this enzyme accumulates. The mutant form of orotidylate decarboxylase blocks the remainder of the pyrimidine biosynthesis pathway, preventing further products from repressing ATCase synthesis.

The large amounts of ATCase that can be isolated from this mutant of E. coli have allowed detailed study of the enzyme by the various techniques of enzymology and protein chemistry.

1.3.3. KINETIC AND PHYSICAL PROPERTIES OF ATCase

(a) KINETIC PROPERTIES OF ATCase

The rate of reaction of pure ATCase in the presence of saturating carbamoyl phosphate shows a sigmoidal dependence on the aspartate concentration (Gerhart & Pardee, 1962) as shown in Fig. 1.3. The effect of the heterotropic feedback inhibitor CTP and the activator ATP is also illustrated. ATP added to a CTP inhibited system can reverse the observed heterotropic inhibition. This feature allows the "fine-tuning" of the catalytic activity of native ATCase.

(b) DESENSITIZATION OF ATCase

Treating ATCase with various mercurials or

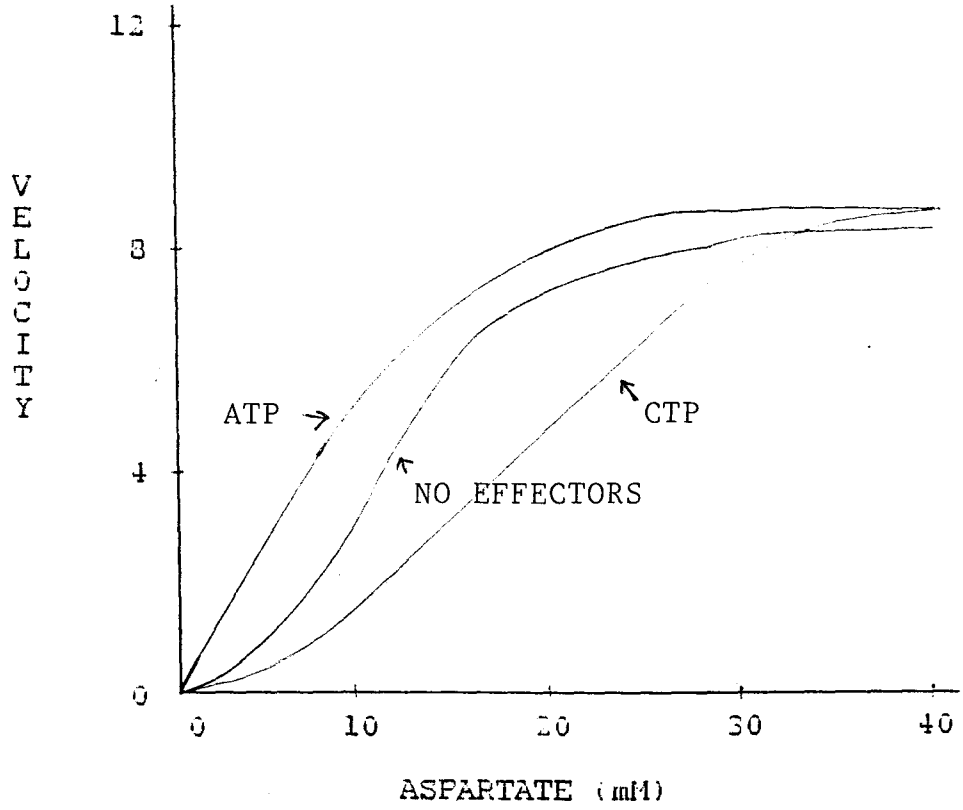


FIGURE 1.3 -- Inhibition velocity profile of ATCase. The velocity was measured at pH 7.0, in the presence and absence of allosteric effectors. Velocity is reported in units of mmole of carbamoyl aspartate formed per hour per mg of enzyme (Kantrowitz et al., 1980a).

heating it to 60°C at low ionic strength removes the sensitivity of the enzyme to CTP inhibition (Gerhart & Pardee, 1962). This new form of the enzyme showed an initial velocity profile with respect to aspartate that was hyperbolic rather than sigmoidal. This desensitization to the CTP inhibition, thought to be due to dissociation of the enzyme (Gerhart & Pardee, 1964), led to further study on the subunit structure of ATCase.

(c) ELUCIDATION OF THE SUBUNIT STRUCTURE OF ATCase

The desensitization of ATCase by treatment with parahydroxymercuribenzoate (pHMB) was found to give rise to two different subunit species (Gerhart & Schachman, 1965). The larger of the subunit species was found to be catalytically active. However, it showed simple hyperbolic initial velocity kinetics and was insensitive to the heterotropic inhibitor CTP. This subunit species was termed the catalytic subunit and is the subject of this research. The smaller of the subunit species was found to bind the inhibitor CTP but showed no catalytic activity (Changeux et al., 1968). This species has been termed the regulatory subunit. By removing the mercurial causing dissociation, the catalytic and regulatory subunits were found to spontaneously reassociate to form an aggregate having all the properties of native ATCase (Gerhart & Schachman, 1965).

At this time, accurate values for the molecular weights of ATCase, the constituent subunits and their

individual polypeptide chains and consequently the number of each chain in the active enzyme, were unknown. In 1968 the complete sequence of the regulatory subunit was published (Weber, 1968), and a molecular weight of 17,000 was calculated for this peptide. Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis results verified the molecular weight of the regulatory peptide and gave a value of approximately 33,000 for the catalytic chain. This indicated that the native enzyme was an aggregate of six catalytic and six regulatory chains. X-Ray crystallographic analysis revealed that the enzyme molecule had both a two fold (Steitz et al., 1967) and a three fold axis of symmetry (Wiley & Lipscomb, 1968).

The complete amino acid sequence of the catalytic subunit was published in 1983 (Konigsberg & Henderson, 1983). It is composed of three 34,000 M.W. chains and will be abbreviated as c_3 while the regulatory subunit is a dimer (r_2) of 17,000 M.W. chains. Native ATCase (c_6r_6, M.W. 310,000) is constructed of two catalytic subunits interposed by three regulatory subunits. This model for the arrangement of the subunits was based on work from many groups (Warren et al., 1973; Wiley et al., 1971; Richards & Williams, 1972; Rosenbusch & Weber, 1971a; Markus et al., 1971).

(d) ZINC AND THE REGULATORY SUBUNIT

The native enzyme was found to contain six zinc ions (Gerhart, 1970; Rosenbusch & Weber, 1971a) which are located in the regulatory subunits, one per polypeptide chain (Rosenbusch & Weber, 1971b). The zinc binding site in the native enzyme is near the catalytic subunit but distant from the CTP binding site (Fan et al., 1975; Matsumato & Hammes, 1975; Griffin et al., 1973). Therefore the zinc ions are involved in the association of c_3 with r_2 but not with the binding of the effector molecules CTP and ATP.

(e) CATALYTIC SUBUNIT

The isolated catalytic subunit is insensitive to allosteric effectors. This property allows the kinetic properties of the enzyme to be studied in the absence of the regulatory effects the native enzyme would impose, thus simplifying the kinetic analysis. The kinetic properties of the catalytic subunit are slightly different than the native enzyme: the maximum velocity (V_{max}) and Michaelis constant (K_m) are greater for catalytic subunit (Evans et al., 1974).

1.3.4. ACTIVE SITE OF C_3

The active site of E. coli aspartate transcarbamoylase consisting of four different regions (Fig. 1.7) has been studied using substrate analogues.

(a) DICARBOXYLIC ANALOGUES

Several competitive inhibitors of L-aspartate have been used to study the substrate's structural features required for function and tight binding. These analogues were found to bind with dissociation constants (K_i's) greater than 1mM. It was observed that 4-carbon dicarboxylic acids were bound with the highest affinity, succinate binding tighter than either malonate or glutarate (Porter et al., 1969). The carboxylates have been proposed to be in approximately a cis conformation; maleate and succinate are bound with essentially the same affinity while fumarate is a very poor ligand (Porter et al., 1969). The addition of an α-substituent decreases the inhibitor's affinity relative to succinate (Porter et al., 1969). This at first appears to be an anomaly for the natural substrate, L-aspartate, contains an α-group. This is the site of reaction, the mode of steric hindrance being used to enhance catalysis (Section 1.3.6.3.(e)). A second substituent, either at the α-position or at the β-threo position abolishes binding (Davies et al., 1970). Analogues substituted at the β-erythro position retain the parent compound's tight binding, erythro-β-hydroxyl-L-aspartate being an excellent substrate (Davies et al., 1970).

(b) PHOSPHATE AND CARBOMOYL PHOSPHATE ANALOGUES

Many competitive inhibitors of carbamoyl phosphate have been employed to determine the features of

this substrate which contribute to activity and tight binding. All compounds containing a free phosphate or phosphonate moiety were found to bind quite tightly; the dissociation constants were all less than 1.5mM (Porter et al., 1969). This indicates that the phosphate binding region is freely accessible to the solvent. The amino group of carbamoyl phosphate is not required for activity, both acetyl phosphate and N-methylcarbamoyl phosphate can act as substrates (Porter et al., 1969).

(c) BISUBSTRATE ANALOGUES

Three inhibitors combining features of both substrates have been synthesized and tested; phosphonoacetyl-L-aspartate (PALA) (Collins & Stark, 1971), 4,5-dicarboxy-2-ketopentyl phosphonate (DIKEP) (Swyryd et al., 1974), and 4,5-dicarboxy-2-hydroxypentyl phosphonate (DIHOP) (Roberts et al., 1976) (see Fig. 4.2). The best, PALA, is the most tightly bound reversible inhibitor of the E. coli enzyme so far discovered (K_i=27nM pH 7.0 28°C). Its methylene analogue, DIKEP, was found to bind 10 fold poorer. This difference could be attributed to either the change in polarizability of the carbonyl (a ketone versus an amide in PALA), the presence of the amide nitrogen in PALA, or a combination of these two features (Roberts et al., 1976). The alcohol obtained by reduction of DIKEP, DIHOP, is quite a poor ligand (K_i~4μM pH 7.0 28°C) considering the presence

of both a phosphate and succinate moiety (Roberts et al., 1976).

1.3.5. ACTIVE SITE RESIDUES

The use of the above analogues to protect against modification reagents has led to the identity of many residues at or near the active site. Recently, the solving of the 3.0Å X-ray crystallographic map of the enzyme has implicated other residues in the environment of the active site (Honzatko & Lipscomb, 1982).

(a) CYSTEINE

Chemical modification of the lone cysteine residue ($R=-CH_2-SH$) of the catalytic chain (Cys47) has indicated that it is not necessary for substrate binding or activity. However, since the small S-cyano derivative retains full activity (Vanaman & Stark, 1970) while the bulky derivatives (modified by p-hydroxymercuribenzoate (Vanaman & Stark, 1970), 5,5'-dithiobis(2-nitrobenzoate) (Vanaman & Stark, 1970) or 2-chloromercuri-4-nitrophenol (Evans et al., 1972)) are all inactive. Fluorescence energy transfer experiments have put this cysteine in close proximity to the active site (Matsumoto & Hammes, 1975). The preferential reaction of this residue with negatively charged reagents such as permanganate (Benisek, 1971) and 2-chloromercuri-4-nitrophenol (Evans et al., 1972), indicates that it is near a positively charged group on the

enzyme.

(b) ARGININE

Arginine, an amino acid which has a positively charged side chain ($R=-CH_2-CH_2-CH_2-NH-C(NH_2)NH_2^+$), has been found to be important in the binding of the substrates to the active site. There are 12-13 arginines in each catalytic chain (Konigsberg & Henderson, 1983). The loss of activity upon treatment with phenylglyoxal can be directly correlated with the modification of one arginine residue at the active site (Kantrowitz & Lipscomb, 1976). Protection experiments involving substrates and substrate analogues have placed the essential arginine at the carbamoyl phosphate binding site. X-Ray crystallographic studies have shown that Arg154, Arg105, and Arg165 form three salt links with the carbamoyl phosphate analogue, pyrophosphate (Honzatko & Lipscomb, 1982) (see Fig. 1.4).

(c) HISTIDINE

Photo-oxidation of the Schiff's base formed between arginine and phenylglyoxal caused the modification of two histidines which, therefore, must be near the active site (Greenwell et al., 1973). Bromosuccinate is capable of modifying one of these histidines, affecting activity (Gregory & Wilson, 1971). His134 has been found to be within 4Å of an oxygen of bound pyrophosphate (Honzatko &

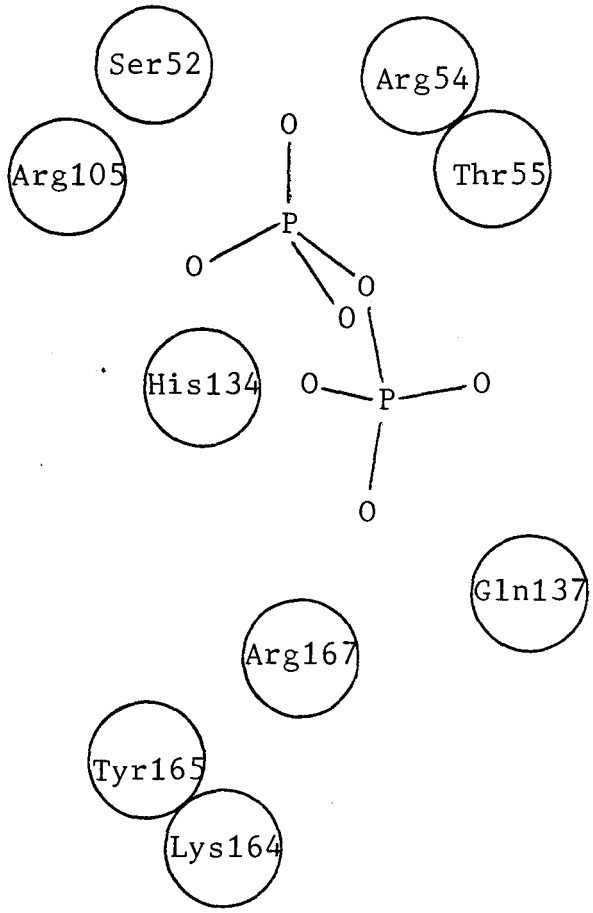


FIGURE 1.4 -- Spatial relationship of pyrophosphate to residues of the catalytic chain in the phosphate crevice. His134 (edge-on to 1 of the phosphates of pyrophosphate), Ser52 and Thr55 are sufficiently close to form hydrogen bonds with the ligand. The most prominent interactions of pyrophosphate involve arginine residues 54, 105 and 167 (schematic representation of Fig. 3, Honzatko & Lipscomb, 1982).

Lipscomb, 1982). This residue is in van der Waals' contact and edge-on with respect to one of pyrophosphate's phosphate units (see Fig. 1.4).

(d) TYROSINE

Of the eight tyrosine residues ($R=-CH_2-C_6H_4-OH$) per catalytic chain, tetranitromethane has been found to react with two residues (Kirschner & Schachman, 1973; Evans et al., 1975). Nitration of only Tyr165 was correlated with loss of enzyme activity. This residue was placed in the vicinity of the carbamoyl phosphate binding site by substrate protection studies (Lauritzen et al., 1980). From the X-ray crystallographic map, this residue is well over 10Å from the phosphate binding site (Honzatko & Lipscomb, 1982) (see Fig. 1.4).

(e) LYSINE

There are sixteen lysines ($R=-CH_2-NH_3^+$) in each catalytic chain. Six of these were found to react with succinic anhydride yielding an inactive enzyme that could still bind carbamoyl phosphate but not aspartate or its analogues (Meighen et al., 1970). When the catalytic subunit was treated with pyridoxal phosphate (not pyridoxal alone) a Shiff's base was found to form with Lys83 (Kempe & Stark, 1975). It was thought that the phosphate of this reagent was bound within the phosphate region while the pyridoxal group "stretched" across to react within the

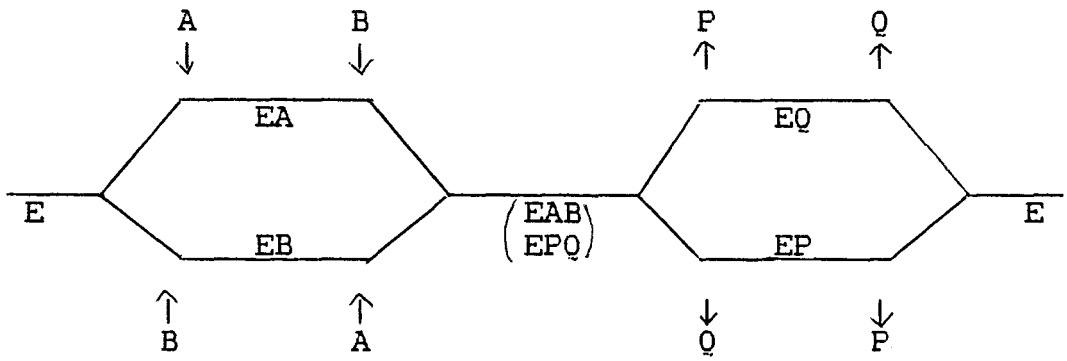
dicarboxylate region. Lys83 as well as Lys84 and Lys224 have been reported to be alkylated by bromosuccinate (Lauritzen & Lipscomb, 1982) resulting in a loss of enzymatic activity. Again, various inhibitors that bind to the active site were found to give varying degrees of protection. It appears that these three lysine residues are at or near the active site (Fig. 1.4).

1.3.6. CATALYTIC MECHANISM OF C_3

1.3.6.1. ORDERED VERSUS RANDOM MECHANISM

Two different types of mechanism have been proposed for the E. coli enzyme. Heyde and coworkers (Heyde, 1976) have proposed a rapid equilibrium mechanism with three strongly bound dead-end complexes (Fig. 1.5) while Stark and others (Jacobson & Stark, 1975) have argued that the enzyme follows an ordered BiBi mechanism (Fig. 1.6).

Most of the available evidence supports an ordered BiBi mechanism. In product inhibition studies, carbamoyl-L-aspartate is a competitive inhibitor of L-aspartate but noncompetitive versus carbamoyl phosphate (Porter et al., 1969). Phosphate is a competitive inhibitor of carbamoyl phosphate but noncompetitive versus L-aspartate (Porter et al., 1969). Product inhibition studies, though, are not conclusive. The question is not whether an enzyme-L-aspartate complex forms, but whether it is a productive complex. The inhibition of the catalytic subunit by PALA is competitive with respect to carbamoyl phosphate and



DEAD-END COMPLEXES:

E*(aspartate)*(phosphate)
E*(aspartate)*(carbamoyl aspartate)
E*(carbamoyl phosphate)*(carbamoyl aspartate)

FIGURE 1.5 -- Rapid equilibrium mechanism with three strongly formed dead-end complexes.

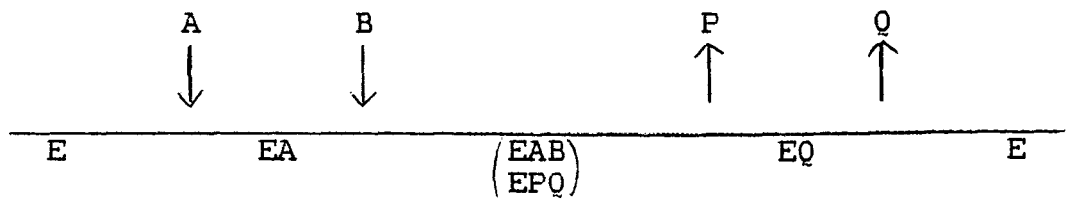


FIGURE 1.6 -- Ordered BiBi mechanism.

noncompetitive with respect to L-aspartate (Collins & Stark, 1971). The latter inhibition is affected equally by the same high concentration of L-aspartate and lysine-acetate (spectrophotometric experiments by Jacobson and Stark (1975)) and in kinetic experiments both compounds inhibit the binding of PALA equally (and to a greater extent then they inhibit the binding of carbamoyl phosphate) (Jacobson & Stark, 1975). Therefore, the failure of L-aspartate to relieve inhibition by PALA at constant ionic strength suggests that the enzyme-L-aspartate complex must be unproductive.

In further support of the ordered BiBi mechanism, isotope exchange kinetic experiments showed that the [^{14}C]-L-aspartate $\rightleftharpoons$ carbamoyl-L-aspartate exchange was much faster than the [^{32}P]-carbamoyl phosphate$\rightleftharpoons$phosphate exchange (Wedler & Gasser, 1974).

Recent X-ray crystallographic results (Honzatko & Lipscomb, 1982) have shown a second false binding site for the dicarboxylic acid moiety of PALA. The existance of two binding sites for dicarboxylic acids far removed from each other lends some support to Heyde's interpretation of the kinetic data.

1.3.6.2. DIRECT TRANSFER OF CARBAMOYL GROUP

Several lines of evidence support the hypothesis that the carbamoyl group of carbamoyl phosphate is

transferred directly to L-aspartate without the formation of either cyanate or a carbamoyl-enzyme intermediate. At neutral pH, carbamoyl phosphate decomposes to yield cyanate and phosphate (Allen & Jones, 1964). It is unlikely that cyanate could be the form of the transferred group since acetyl phosphate is also a good substrate for ATCase (with a very similar catalytic mechanism) (Porter et al., 1969; Heyde & Morrison, 1973) and acetyl phosphate cannot yield a ketene by a mechanism similar to that which results in cyanate from carbamoyl phosphate. In the absence of L-aspartate, the enzyme does not catalyse the decomposition of carbamoyl phosphate as determined from isotope exchange experiments with and without succinate, a competitive inhibitor of L-aspartate (Porter et al., 1969). Also, the kinetic data of Porter et al. (1969) conclusively rules out a ping-pong mechanism: phosphate is a competitive inhibitor for carbamoyl phosphate, and therefore the formation of a carbamoyl-enzyme intermediate with a dissociable phosphate cannot occur. A carbamoyl-enzyme intermediate in the ternary enzyme-carbamoyl phosphate-L-aspartate complex has not been eliminated.

1.3.6.3. EVIDENCE FOR MECHANISTIC PROPOSALS

(a) NH_2 GROUP OF CARBAMOYL PHOSPHATE NOT REQUIRED FOR ACTIVITY

The amino group of carbamoyl phosphate is not required for catalytic activity. The maximum rates of

reaction (V_{max}) for acetyl phosphate and N-methylcarbamoyl phosphate are 2.4% and 0.03%, respectively (compared to that of carbamoyl phosphate). N,N-dimethylcarbamoyl phosphate is not a substrate, implying that the size of the substituent adjacent to the carbonyl group is much more important than its chemical nature (Porter et al., 1969).

(b) CONFORMATION CHANGE UPON FORMATION OF C_3-CARBAMOYL PHOSPHATE COMPLEX

Upon binding of carbamoyl phosphate to the catalytic subunit, a conformation change is observed. This conformation change can be monitored by optical rotary dispersion (ORD) (Pigiet, 1971) or circular dichroism (CD) (Griffin et al., 1972).

(c) INTERACTIONS AT CARBONYL OXYGEN OF CARBAMOYL PHOSPHATE

Experimental evidence indicates an interaction at the carbonyl oxygen of carbamoyl phosphate. Upon the formation of the carbamoyl phosphate-enzyme complex there is a small change in the ^{13}C-NMR chemical shift of the carbonyl carbon resonance (2Hz upfield at pH 7.0) (Roberts et al., 1976). When succinate is added, forming the ternary complex, there is a large downfield change in the chemical shift for carbamoyl phosphate (17.7$\pm$1.0Hz) consistent with the interaction between the carbonyl group and a proton donor on the enzyme. PALA and DIKEP also induce this

downfield shift. Carbamoyl phosphate analogues that have been found not to induce a conformation change of the enzyme (e.g. phosphonacetamide), do not induce the chemical shift change. This interaction could be a partial protonization or deformation of the carbonyl group.

(d) CONFORMATION CHANGE UPON FORMATION OF C_3-CARBAMOYL PHOSPHATE-4-CARBON DICARBOXYLIC ACID

A second conformation change of the enzyme occurs when a 4-carbon dicarboxylic acid binds to the enzyme-carbamoyl phosphate complex in approximately a cis configuration. This conformation change is not just an enhancement of the effect when carbamoyl phosphate binds to the free enzyme for the CD difference spectra shows a different sign (Griffin et al., 1972). The sedimentation coefficient of the ternary complex increases by 1.05% over that of the binary complex (Kirschner & Schachman, 1971). Also, the ultra-violet (UV) spectra of the ternary complex with succinate is greater than that of the two binary complexes, enzyme-carbamoyl phosphate plus enzyme-succinate (Collins & Stark, 1969).

(e) COMPRESSION

There have been several lines of evidence that have substantiated a compression between the amino group of L-aspartate and the carbonyl carbon of carbamoyl phosphate being important in catalysis. The conformation

change when succinate is added to the enzyme-carbamoyl phosphate complex is opposed by analogues with bulky L-α-substituents (e.g. L-malate and α-methylsuccinate) (Hammes et al., 1971; Collins & Stark, 1969). The binding of L-aspartate and L-malate is enhanced by phosphate while the binding of succinate is not (Collins & Stark, 1969). The van der Waals repulsion between the nitrogen and carbon atoms opposes their close approach and contributes to the large activation energy barrier to reaction. Compression of the two substrates together along the reaction coordinate in the direction of the transition-state would clearly help to lower the activation energy and hence would accelerate the reaction (Jencks, 1969).

1.4. DESIGN OF RESEARCH

Conceptually, the active site of ATCase may be divided into four regions according to their interaction with substrate molecules (Fig. 1.7). The extensive work of Stark and others has clearly delineated the dicarboxylate and phosphate regions. The former has a strong preference for a four-carbon dicarboxylic acid in the cis conformation. The latter region has a general requirement for a phosphate or phosphonate moiety. In addition, the amino region is known to exert a severe steric hindrance on substituents in this position. However, relatively little information is

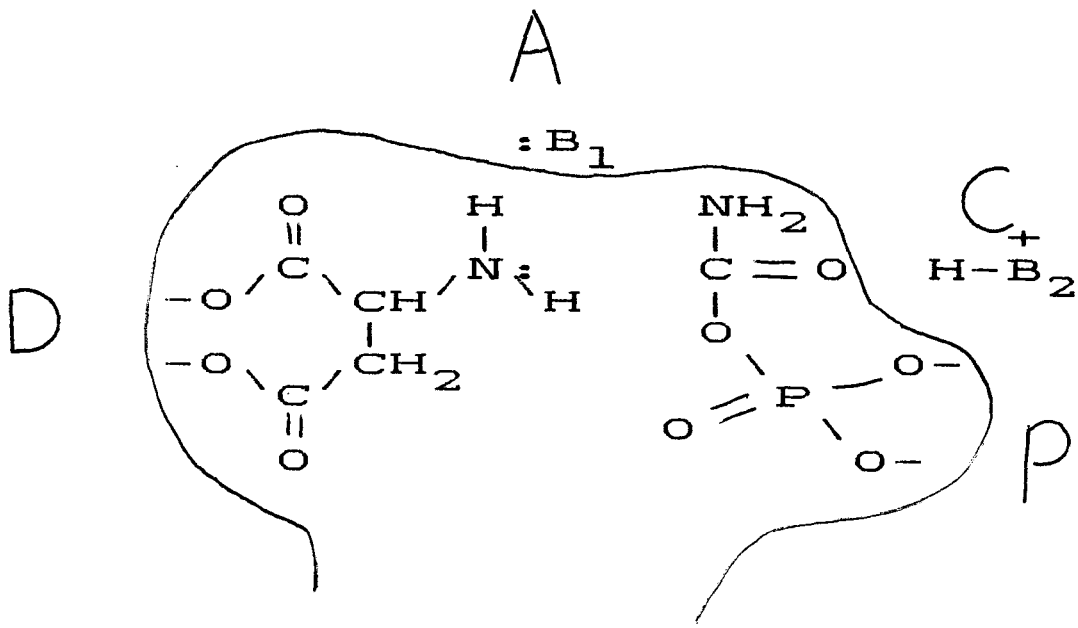


FIGURE 1.7 -- Diagramatic representation of the active site of ATCase. The letters donate regions referred to in the text: A, amino; C, carbamoyl; D, dicarboxylate; and P, phosphate.

available concerning the carbamoyl region in spite of its obvious relevance to the catalytic mechanism.

The object of the present study is to probe the active site of the catalytic subunit of E. coli ATCase using a variety of synthetic as well as commercially available compounds. The outcome of this research will further outline the catalytic mechanism and it is hoped that this knowledge will lead to specific and potent inhibitors that may be of pharmacological interest.

In the past, enzyme inhibitors were often designed to appear like the natural substrates. However, this approach had only limited success for several reasons. A given substrate may be metabolized by several enzymes. Also, enzymes usually do not show a very high affinity for their substrates, the extra binding energy being utilized to promote the reaction by the formation of the transition state or to overcome the loss of entropy (see Section 1.1.3.). The intrinsic binding energy betwwen a protein and a small ligand can be extremely high (Jencks, 1975). When not only the structure of the substrates but the mechanism of the reaction are considered, extremely tight binding inhibitors (the K_is often in the 10^{-9}M range) can be created. The inhibitors which resemble the putative transition state also show a very high degree of specificity since enzymes which have the same substrate have distict transition state (Wolfenden, 1976). By using the results

from this research, it was hoped that a tight binding transition state analogue with a very high degree of selectivity could be designed and tested.

It is within the carbamoyl region of ATCase that the chemical reaction occurs. Therefore, for a complete understanding of the catalytic mechanism, it is necessary to investigate the role of geometric or electrostatic destabilization of the substrates occurring within this region. By drawing upon the body of knowledge for similar chemical and enzymatic reactions, a prediction as to the structure of the transition state can be made.

In the reaction catalysed by ATCase, the trigonal acyl group of carbamoyl phosphate is transferred to the amine of aspartate. The acyl group of the product molecule, N-carbamoyl-L-aspartate, retains the trigonal geometry of the donor molecule, carbamoyl phosphate. In many similar chemical reactions, the reaction proceeds through a tetrahedral transition state. For example, the transfer of the acetyl group from the acetylimidazolium ion to a primary amine (Fig. 1.8) proceeds through such a tetrahedral transition state (Page & Jencks, 1972). Enzyme catalysed reactions are also known to stabilize a tetrahedral transition state (eg. glutamine synthetase (Jencks, 1975)).

The attack of a nucleophile upon a susceptible group has been found to be more favourable if the electrophile becomes more polarized. Teshema et al. (1982) showed a

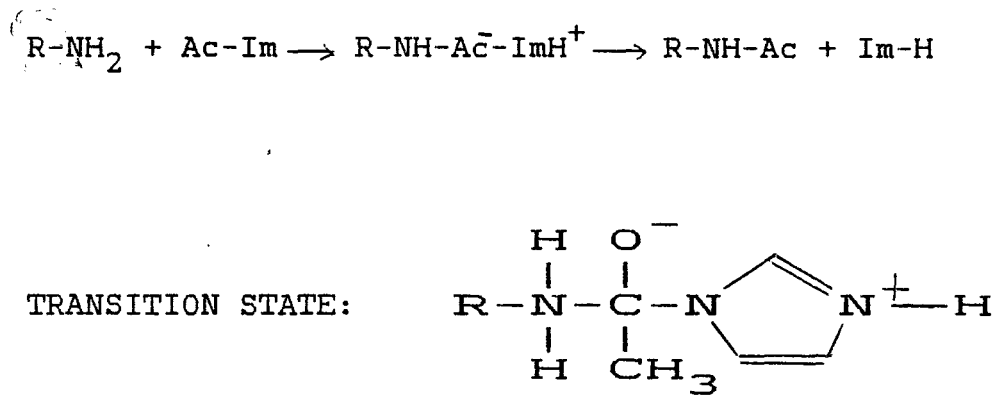


FIGURE 1.8 -- Transfer of acetyl group from the acetyl-imidazole to a primary amine. The abreviations used are: Ac, acetyl group; and Im, imidazolium group (a good leaving group much like the phosphate group of carbamoyl phosphate (from Page & Jencks, 1972).

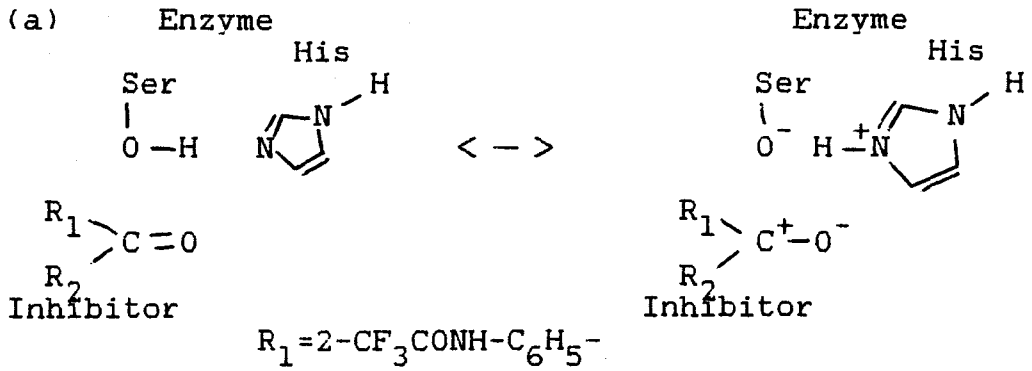
correlation between the carbonyl-stretching frequency of various inhibitors of the serine proteases and their pK_i. As the carbonyl stretching frequency increased, the binding of the inhibitors increased. Therefore, as the carbonyl group of the inhibitor becomes more polarized, it becomes more succeptible to nucleophilic attack (Fig. 1.9). In the case of ATCase, there is a very similar reaction. The amino group of aspartate (nucleophile) attacks the carbonyl carbon of carbamoyl phosphate to effect transfer.

By applying the previous two lines of research to the enzyme ATCase, it may be predicted that the mechanism of the reaction proceeds as drawn in Fig. 1.10. The tetrahedral anionic transition state drawn, has been postulated for many years (Stark, 1971) but prior to this work, no research has been done to prove or disprove this theory.

With a knowledge of the enzyme mechanism, very potent irreversible reagents may be designed. Previously, a reactive group was simply placed at a strategic location on the inhibitor and substrate recognition was used to bring this molecule to the active site. The so-called suicide or k_{cat} inhibitors on the other hand, are intrinsically unreactive but are transformed by the catalytic action of the enzyme into chemically reactive species (Abeles & Maycock, 1976) resulting in a high degree of specificity.

Since the catalytic mechanism of ATCase was known in

(a)



R_1=2-CF_3CONH-C_6H_5-

(b)

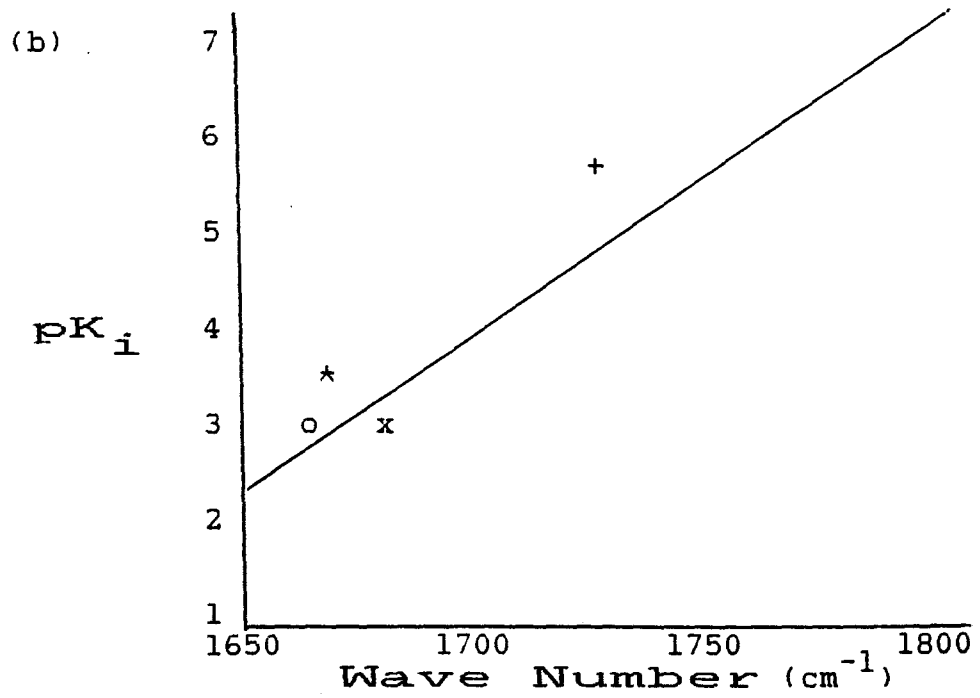


FIGURE 1.9 -- Correlation between the polarizability of the carbonyl group and the affinity of an inhibitor for serine proteases. The data was taken from Teshema et al. (1982).
(a) Schematic diagram showing the binding of inhibitor within active site.
(b) Graph showing correlation between the carbonyl-stretching frequency (a measure of polarizability) of various inhibitors and their pK_i. The compounds plotted are: o, R_2=CH_3; ★, R_2=$NHCH_2C_6H_5$; x, R_2=$OCH_2C_6H_5$; and +, R_2=$SCH_2C_6H_5$.

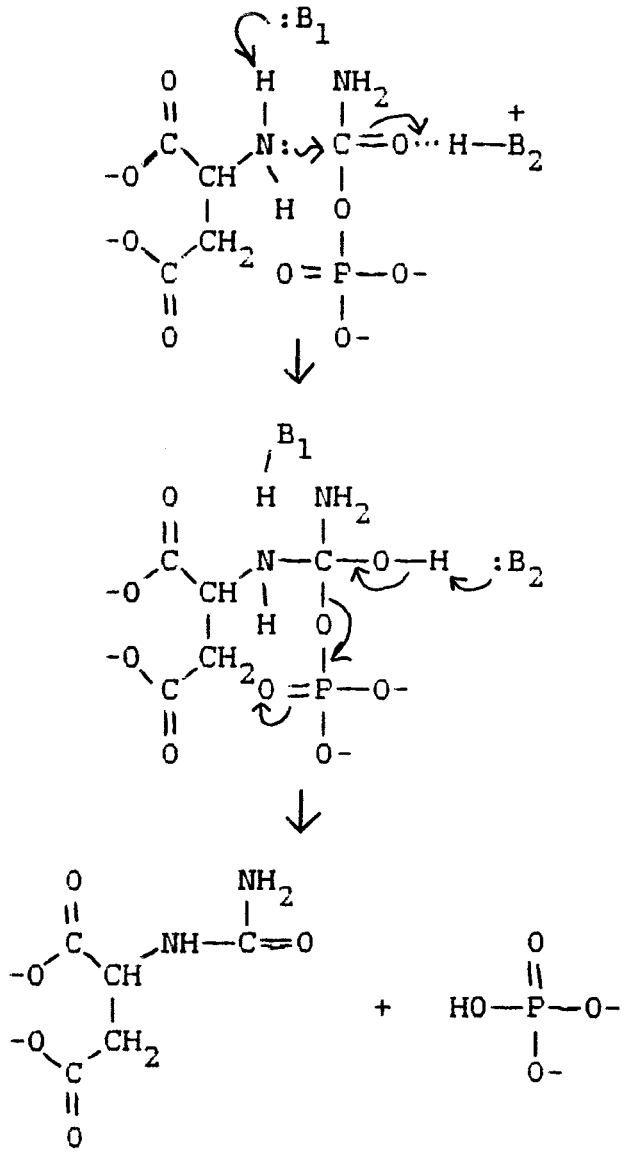


FIGURE 1.10 -- Predicted reaction mechanism of ATCase.

some detail, it was felt that a suicide inhibitor could be designed. The position of the postulated base B_1 and the proton donor HB_2 (Fig. 1.7) indicated that a properly designed series of proton transfers would yield a highly reactive species within the active site. The potential suicide inhibitor 3-nitro-2-cyanomethylacrylic acid was designed to meet these requirements as well as having enough similarity to the natural substrate L-aspartate to be held within the active site in a proper orientation. This compound has an acidic proton corresponding to the amino region of the normal substrate (Fig. 1.11) Abstraction of this proton by the postulate base (B_1) in the enzyme should lead to the highly reactive keteneimine especially since a proton donor (HB_2) should be in the carbamoyl region. The now deprotonated nucleophile B_2 can add to the activated double bond.

In summary, a more detailed study of the catalytic mechanism of ATCase has been undertaken. It is hoped that a by-product of this research may be the development of a potent and specific inhibitor yielding the desired pharmacological activity.

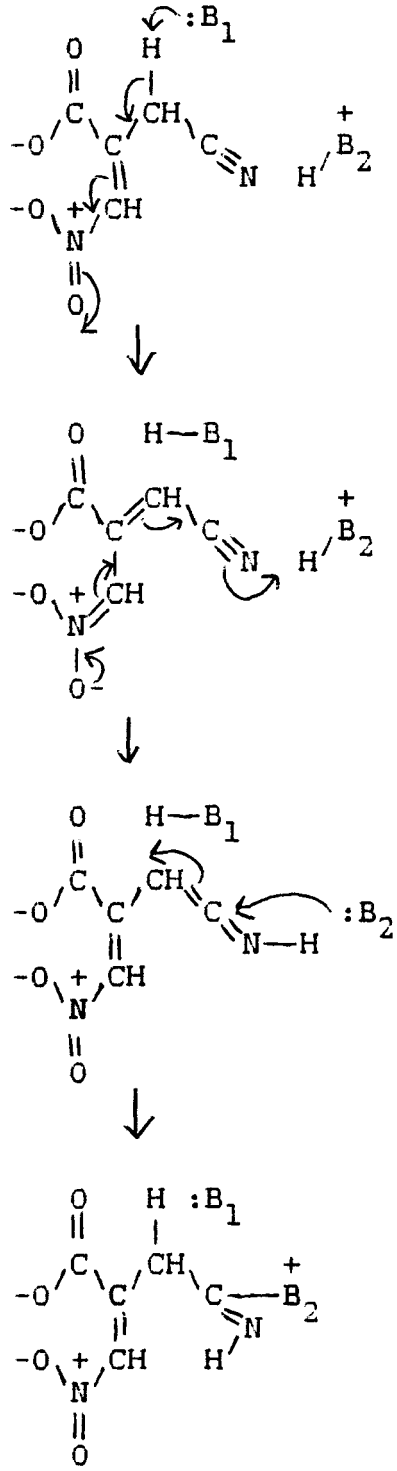


FIGURE 1.11 -- Predicted mechanism of action of 3-nitro-2-cyanomethyl acrylate.

2. MATERIALS and METHODS

2.1. MATERIALS

[^{14}C]-L-Aspartic acid (233.0mCi/mmole) was obtained from New England Nuclear (Lachine) and purified by absorption to cation exchange resin (Amberlite CG-120, 100 to 200 mesh) as described by Porter et al. (1969). The purified [^{14}C]-L-aspartic acid was diluted with unlabelled potassium aspartate to a specific activity of 28.3mCi/mmole and stored at -20°C.

[^{14}C]-Carbamoyl phosphate (10.4mCi/mmole) was obtained from New England Nuclear (Lachine), diluted with unlabeled dilithium carbamoyl phosphate in 200mM sodium formate buffer (pH 3.8) and stored in aliquots at -70°C. The final concentration of carbamoyl phosphate in these aliquots was 50mM (3.1mCi/mmol) for stability reasons (Allen & Jones, 1964).

Disodium hydrogen orthophosphate, sodium carbonate, potassium carbonate, sodium lauryl sulfate, boric acid, bromocresol green, ethylenediaminetetraacetic acid (EDTA), PPO (2,5-diphenyloxazole), POPOP (p-bis[2-(5-phenyloxazolyl)]benzene, and sodium hydroxide were obtained from BDH Chemicals Canada Ltd. (Toronto). Methylenediphosphonic acid, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), sulfur trioxide pyridinium complex (technical

grade), dibenzylphosphite (technical grade), diphenylphosphochloridate, N-chlorosuccinimide, 1,2,3,4-butanetetracarboxylic acid, 1,2,4,5-benzenetetracarboxylic acid, tetrahydrofuran 2,3,4,5-tetracarboxylic acid (a mixture of diasteriomers), 1,2,4-benzenetricarboxylic acid, 3,4-furandicarboxylic acid, 4-sulfophthalic acid, 1,2,3,4-cyclopentanetetracarboxylic acid (all cis), 10% Palladium/carbon, diethylbromoethylphosphonate, diethylphosphonacetaldehyde diethylacetal, and malachite green oxalate were purchased from Aldrich Chemical Co.(Milwaukee). N-Acetyl-L-aspartate, N-formyl-L-aspartate, N-carbamyl-DL-aspartate, N-methyl-L-aspartate, N-formimino-L-aspartate, succinic acid, trans-aconitic acid, cis-aconitic acid, tricarballylic acid, phthallic acid, 2-aminoethylphosphonic acid, aminomethylphosphonic acid, phosphonoacetic acid, phosphonoformic acid, dilithium carbamoyl phosphate, monomethylphosphate, L-aspartate, D-aspartate, threo $D_s(+)$ isocitrate, L-glutamate, bovine serum albumin, anthranilic acid, ascorbic acid, methanesulfonylchloride, cacodylic acid, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonate (HEPES), picrylsulfonic acid (Grade 1), itaconic acid, thioacetic acid, dicyclohexylamine, and bromotrimethylsilane were obtained from Sigma Chemical Co. (St. Louis). Phosphonoacetyl-L-aspartate (PALA) (lot KM22-61-1) was a kind gift of the National Cancer Institute (Bethesda).

Solvents were Baker grade.

2.2. ENZYME ISOLATION AND ASSAY

Aspartate transcarbamoylase was isolated by Duncan K.K. Chong using the method of Gerhart and Holoubek (1967) and stored in 40mM potassium phosphate buffer (pH 7.0) containing 2mM 2-mercaptoethanol and 0.2mM EDTA. The subunits were separated by reaction with neohydrin followed by DEAE-Sephadex chromatography (Schachman, 1972). Preparations of the catalytic subunit were stored in 40mM potassium phosphate buffer (pH 7.0) containing 2mM 2-mercaptoethanol and 0.2mM EDTA.

Protein concentrations were generally determined from the absorbance at 280nm. Values of absorbance for 1 mg/mL solutions (1cm path length) were taken as 0.59 for aspartate transcarbamoylase, 0.72 for the catalytic subunit, and 0.32 for the zinc regulatory subunit. Protein measurements were also made using the methods of Lowry et al. (1951) and this method was standardized against the absorbance procedure (Dennis et al., 1985).

2.2.1. [^{14}C]-L-ASPARTATE ENZYME ASSAY

This assay was a modification of the procedure of Bresnick and Mosse (1966), Porter et al. (1969), and Mort and Chan (1975). The reaction was conducted in cacodylate

buffer (0.1M, pH 7.5) containing bovine serum albumin (50μg/mL) together with labelled aspartate (100μM, 10^3cpm/nmole, 50,000cpm/assay) and inhibitor (adjusted to pH 7.5). The enzyme was preincubated at 25°C in this buffer for 15 minutes, with the reaction initiated by the addition of carbamoyl phosphate (80 to 350μM) and terminated after 10 minutes with 100μL of 1M phosphoric acid. The reaction mixture (final pH 2.5) was then applied to Pasteur pipette columns containing Amberlite CG-120 (100 to 200 mesh) in the H^+ form and the columns were washed twice with 1mL aliquots of 0.2M acetic acid. The eluant and washings were collected directly in scintillation vials. Without further treatment, 20mL of the scintillation fluid described by Anderson and McClure (1973) (Triton X-114 and xylene (1:3, v/v) containing 3g of PPO (2,5-diphenyloxazole) and 0.2g of POPOP (p-bis[2-(5-phenyloxazolyl)]benzene) per liter) were added to the samples and radioactivity was determined on a Beckman LS-230 Liquid Scintillation Counter. The measured counts per minute were corrected for the background due to impurities in the labelled aspartate. This background amounted to 0.5 to 1.0% of the counts in freshly purified [^{14}C]-L-aspartate and tended to increase upon storage. The assays were linear under these conditions.

Attempts were made to determine the inhibition constants according to Dixon (1953). The resultant plots did not yield interpretable graphs (the intersection point

on each graph was displaced to the right of the ordinate). To overcome this unusual behavior, several features of the assay were varied: incubation time; substrate concentrations; initiating species; assay time course; overall ionic strength; and buffering species. In no case could the unusual behavior be overcome or identified. It was possible however, to determine approximate binding constants from IC_{50} studies but these were not of sufficient accuracy for the intended study. Also, IC_{50} studies do not give any information concerning the nature of inhibition (competitive, noncompetitive, or uncompetitive).

2.2.2. [^{14}C]-CARBAMOYL PHOSPHATE ENZYME ASSAY

This radioactive assay was a modification of the procedure of Swyryd *et al.* (1974). Each assay, in a Beckman mini Poly-Q vial, contained 100mM sodium HEPES, (pH 7.5), 15mM sodium aspartate (pH 7.5), bovine serum albumin (50μg/mL), aspartate transcarbamoylase catalytic subunit (1 to 4 ng/mL), and inhibitor (pH 7.5). This mixture was preincubated at 25°C. The reaction was initiated by the addition of [^{14}C]-carbamoyl phosphate (0°C), bringing the final volume to 400μL. After 15 minutes, the reaction was stopped by the addition of 100μL of 50% trichloroacetic acid. The scintillation vials were transferred to a boiling water bath for 5 minutes, after which the removal of [^{14}C]-carbon dioxide was completed by adding small amounts

of crushed solid carbon dioxide to each tube. The samples were then counted in Aqueous Counting Scintillant (Amersham, Arlington Heights, Illinois) on a Beckman LS-230 Liquid Scintillation Counter.

A unit of activity (U) is defined in this work as the amount of enzyme catalyzing the formation of one μmole of N-carbamoyl-L-aspartate per minute (25°C, pH 7.5). To obtain linearity and a suitable signal-to-noise ratio (>4:1) it was necessary to vary the amount of enzyme in the assay to maintain approximately the same level of carbamoyl phosphate consumption (5-25%).

The patterns of inhibition were determined by analyzing the data with double reciprocal plots (Lineweaver & Burk, 1934) while the inhibition constant was estimated according to Dixon (1953). In all cases, four different carbamoyl phosphate concentrations were varied with four different inhibitor concentrations. Each inhibitor was tested in at least two complete series of assays (16 in number). The errors listed for the K_i determinations are the observed ranges (Dennis et al., 1985).

2.3. SYNTHESIS OF INHIBITORS

All the starting materials and solvents used were reagent grade in 98% or higher purity and used without further purification. Unless specified, thin-layer

chromatography (TLC) was on Silica gel G and compounds were visualised by iodine or ultraviolet (UV) light. All compounds containing thiol groups were identified on TLC as bright yellow spots when sprayed with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and developed in pyridine vapour. Compounds containing thioester groups were identified similarly as amber colored spots when developed in ammonia vapour after spraying with DTNB reagent. Compounds containing free amino groups were identified using Ninhydrin spray reagent (dark blue spots). Phosphate esters were identified on TLC by the spray reagent of Hanes & Isherwood (1949). Fluorescein spray reagent was used to identify sulfonic acid group (yellow spots on a salmon pink background). Acidic compounds were identified using a pH spray reagent (acidic compounds give a yellow spot on a pale blue background). Melting points were determined on a Fisher-Johns hot stage apparatus and were uncorrected. Infrared (IR) measurements were carried out on a PE 283 grating spectrophotometer. Liquid samples were measured neat on solid sodium chloride plates and solid samples as a chloroform solution or as potassium bromide pellets. Proton nuclear magnetic resonance (NMR) spectra were recorded on Varian EM-390 spectrometer and chemical shifts (δ) are parts per million (ppm) downfield from tetramethylsilane. DTNB spray reagent was prepared by dissolving 0.5g of DTNB in 25mL of (9:1) methanol:water mixture. Ninhydrin reagent was

prepared fresh daily by adding 25μL of 2,4,6-collidine to 25mL of a 0.2% ninhydrin solution in n-butanol. Fluorescein reagent was prepared by mixing a 10% aqueous silver nitrate solution with 0.2% ethanolic solution of sodium fluorescein in 1:5 ratio. pH Spray reagent was prepared by dissolving 2.5mg of bromocresol green in 25mL of (9:1) acetone:pyridine.

Quantitative amino group analysis was accomplished using trinitrobenzene sulfonic acid (TNBS) by the method of Habeeb (1966). Quantitative phosphate group analysis (Chalvardjian and Rudnicki (1970) with the modification of Duck-Chong (1979)) was performed after 70% perchloric acid digestion (180^{o}C for 0.5h). To 400μL of the sample (1-15nmole) was added 20μL of 1% Triton X-100 in water and 800μL of the assay solution. After incubating for 15 minutes at room temperature, the optical density was measured at 650nm. Calculations were made using a standard curve generated at the same time. The assay solution was made by mixing for 30 minutes, 1 volume of Solution A with 3 volumes of Solution B followed by removal of the fine particles by filtration (good for 2 weeks if stored in the dark). Solution A was a 4.2% solution of ammonium molybdate in 5N hydrochloric acid. Solution B was a 0.2% solution of malachite green in water.

2.3.1. N-METHANESULFONYL-ASPARTATE

To a chilled solution of L-aspartic acid (10g,

76.3mmole) in 2M sodium hydroxide (120mL), was slowly added methanesulfonylchloride (17.5g, 152.5mmole). The reaction was allowed to warm, with stirring, to room temperature. After 3h, the reaction was acidified with concentrated hydrochloric acid (35mL) to pH 1. The mixture was extracted with ethyl acetate 5 X 200mL). The ethyl acetate solution, after being dried with magnesium sulfate, was concentrated, and the product recrystallized from n-propanol-petroleum ether (yield 3.6g or 22%, mp 184-6°C). The NMR spectrum (D_2O) showed d4.4(t,J=6Hz,1H,CH), 3.1(s,3H,-CH_3), and 2.9(d,J=6Hz,2H,-CH_2-). R_f=0.88 (ninhydrin negative) in methylene chloride:methanol:formic acid (80:20:1) (Dennis et al., 1985).

N-Methanesulfonyl-D-aspartate was synthesized in the above manner.

2.3.2. N-SULFO-ASPARTATE and N-SULFO-L-GLUTAMATE

The following synthetic method was adapted from Baumgarten et al. (1932). The sulfur trioxide pyridinium complex (24g, 150.8mmole) was added to a chilled solution of L-aspartic acid (12g, 90.2mmole) and potassium carbonate (40g, 289.4mmole) in water (80mL). The solution was left to stir overnight at 4°C. The precipitate was filtered off and the filtrate extracted with ether (3 X 200mL). A second sulfonation was performed. Potassium carbonate (12g,

86.8mmole) was added to the cool aqueous layer and the solution left overnight. The solution was again filtered and extracted with ether (3 X 200mL). The aqueous layer was concentrated until a precipitate first appeared. The solution was filtered, adjusted to pH 7 with acetic acid, filtered again, and a little 95% ethanol (approximately 1mL) was added to precipitate the inorganic salts. These were filtered and more absolute ethanol was added to the filtrate, with rubbing, until the product crystallized. The product was filtered and washed with ether, then dried in a dessicator. The crystals were tested with ninhydrin spray, if the results were positive for free amine the sulfonation was repeated (the yield was 3.75g or 13%). A TLC (Silica gel G) of the product showed a single spot (R_f 0.24)(iodine vapour or sulfonic acid spray) that was negative to ninhydrin (unless pretreated with acid) in n-propanol:10mM aqueous sodium hydroxide (70:30) as eluent. Amino group analysis with and without hydrolysis (85% phosphoric acid:concentrated sulfuric acid (1:1), 150^{o}C for 1h) showed the product to be greater than 95% pure (Dennis et al., 1985). IR(KBr): 3450, 3000, 1590 (COO^{-}), 1390 (SO_2), 1200 (SO_2) and 1040cm^{-1}.

The synthesis of N-sulfo-D-aspartate was performed as above.

The synthesis of N-sulfo-L-glutamate was performed as per N-sulfo-L-aspartate (R_f=0.30 (iodine vapour)

n-propanol:10mM aqueous sodium hydroxide (70:30)).

2.3.3. N-PHOSPHORYL-L-ASPARTATE

Dibenzyl-L-aspartate was separated from its tosyl salt by extraction into ether from 10% sodium bicarbonate. The ether layer was dried and the solvent removed.

Dibenzylphosphochloridate was synthesized by the method of Atherton (1957). Dibenzylphosphite (6.45g, 24.6mmole) was dissolved in dry benzene (90mL). N-Chlorosuccinimide (3.29g, 24.6mmole) was added and the reaction was stirred at room temperature for 2h. The succinimide was filtered off and the filtrate concentrated on a rotary-evaporator (T<30^{o}C). The result was a viscous yellow oil containing dibenzylphosphochloridate.

The synthesis of N-dibenzylphosphoryldibenzyl-L-aspartate was adapted from Li (1952). Dibenzyl-L-aspartate (7g, 22.3mmole) was dissolved in a mixture of chloroform:triethylamine (75.4mL:7mL). The mixture was cooled to 0^{o}C and the dibenzylphosphochloridate was slowly added in cold chloroform:triethylamine (30mL:0.32mL) with stirring. The reaction was kept at 0^{o}C for 0.5h and then allowed to warm to room temperature. The mixture was stirred overnight. The reaction was washed with water (3 X 100mL), hydrochloric acid (2 X 100mL), 10% sodium bicarbonate (3 X 100mL), and finally water (2 X 100mL). The chloroform solution was concentrated, with the periodic addition of benzene to remove water (yield, 13.3g of yellow

oil). Chromatographic purification over silica gel, gave from methylene chloride:methanol (97:3) as eluents the compound. The product (R_f 0.67, methylene chloride:methanol (95:5) as eluent) was recrystallized (ether-petroleum ether) to a white solid (mp 47-9^{o}C)(yield, 11.0g or 86%). The NMR spectrum ($CDCl_3$) showed δ7.3(s,20H,ArH), 5.0(m,8H,$ArCH_2$), 4.2(t,J=5Hz,1H,CH), 3.9(d,J=10Hz,1H,NH), and 2.9(m,2H,CH_2).

The hydrogenolysis was performed according to the method of Saias & Kornowski (1968). The product was found to be very unstable in aqueous solution. To limit decomposition, the product was dissolved in 10mM sodium hydroxide. To determine the concentration of the inhibitor in the enzyme assay, quantitative amino and phosphate group analyses were performed. Only enzyme assays which showed less than 10% contamination from hydrolysis products are reported (Dennis et al., 1985).

2.3.4. DL-2-SULFOMETHYLSUCCINATE

The synthesis of DL-2-sulfomethylsuccinate was performed by Dr. M.V. Krishna (Dennis et al., 1985). To 1.06g of thioacetic acid (14 mmole) was added 1.3g (10 mmole) of itaconic acid and this mixture was stirred at 80^{o}C for 3h. A yellow oil resulted, from which excess thioacetic acid was removed under reduced pressure to afford a white gummy solid. This solid was triturated with benzene and

recrystallized from benzene to furnish 2-(thioacetylmethyl)succinic acid, 1.48g (72% yield); mp 91-2°C (literature value 90.5-91.5°C, Holmberg & Schjanberg (1940)), R_f=0.75 in methylene chloride:methanol:formic acid (90:10:1) as detected by iodine vapour. NMR ($CDCl_3$): δ2.25(s,3H,methyl) 2.65(d,J=7Hz,2H,CH_2-CO) and 2.8-3.4(m,3H,CH_2 and methine proton).

Hydrolysis of 2-(thioacetylmethyl)succinic acid (0.206g, 1mmole) was carried out at 80°C for 1h in 6mL aqueous 2N sodium hydroxide containing 50% ethanol. After hydrolysis, the contents were acidified with aqueous 3N hydrochloric acid to pH 5 and solvents evaporated to leave a residue which was extracted with ethyl acetate (5 X 10mL). The organic extracts were dried (magnesium sulfate) and solvent was evaporated to give a colourless gum (0.15g) which was identified by TLC, as a mixture of mercaptomethylsuccinic acid and its disulfide with R_f values 0.7 and 0.5 respectively, in methylene chloride:methanol:formic acid (90:10:1).

The gum, as obtained above (0.15g) was directly oxidized with performic acid (freshly prepared from 9.5mL formic acid and 0.5mL of 30% hydrogen peroxide) at room temperature for 12h. The cold mixture was evaporated <u>in vacuo</u> to a small volume and then lyophilized to give a colorless thick gum (0.13g). All attempts to crystallize

this gum were unsuccessful. However, TLC of this material showed a single spot R_f=0.4 in methylene chloride:methanol:formic acid (70:30:1) as detected by both iodine vapour and a spray reagent for sulfonic acids.

This gum (0.13g) was dissolved in a minimum amount of water and neutralised to pH 7 with aqueous 2N sodium hydroxide. A large excess of absolute ethanol was then added to precipitate the trisodium salt of DL-2-sulfomethylsuccinate as a white amorphous solid which was washed with absolute ethanol (6 X 5mL) to furnish a fairly pure sample of the salt, 0.16g (58% yield); mp>300^{o}C; R_f=0.40 in methylene chloride:methanol:formic acid (70:30:1). NMR(D_2O): δ2.6-3.6(m,4H,CH_2-CO and CH_2SO_3) and 3.8(m,1H,methine proton).

2.3.5. 2-MERCAPTOETHYLPHOSPHONATE

The synthesis of 2-mercaptoethylphosphonate was performed by Dr. M.V. Krishna (Dennis *et al.*, 1985). A solution of diethyl 2-bromoethylphosphonate (0.98g, 4mmole) in 20mL ethanol was added to a cooled solution of potassium hydroxide (0.246g, 4.4mmole) and thiolacetic acid (0.38g, 5mmole) in 15mL absolute ethanol with stirring. After overnight incubation, the precipitated potassium bromide was filtered and the clear filtrate was evaporated to leave a yellow oil. This oil was dissolved in 40mL methylene

chloride and washed successively with a saturated sodium bicarbonate solution (2 X 10mL) and water (2 X 10mL). Evaporation of the dried (magnesium sulfate) methylene chloride solution gave a pure sample of 2-(diethylphosphono)ethyl thioacetate as a pale yellow oil, 0.765g (80% yield), R_f=0.5 in methylene chloride:methanol (96:4). IR(film): 1695(thioacetate), 1390, 1240, 1040, 1010 and 950cm^{-1}. NMR($CDCl_3$): δ1.3(t,J=7.5Hz,6H,$\underline{CH_3}$-CH_2), 2.1(m,2H,CH_2-P), 2.3(s,3H,$SCOCH_3$), 3.0(m,2H,CH_2-S), and 4.15(m,4H,CH_3-$\underline{CH_2}$-).

A mixture of 2-(diethylphosphono)ethyl thioacetate (0.26g, 1.083mmole) and 30% hydrogen bromide/acetic acid was heated at 80°C for 3h under anhydrous conditions. Hydrogen bromide and acetic acid were removed <u>in vacuo</u> to leave 2-(phosphono)ethyl thiol as a pale yellow gum, 0.13g. This gum showed a single spot on TLC (PEI cellulose), R_f=0.55 in 0.25M lithium chloride (iodine vapour). The same was detected as a dark yellow spot with DTNB spray reagent when developed in pyridine and as a dark blue spot with Hanes-Isherwood spray reagent. NMR(D_2O): δ1.7-2.2(m,2H,CH_2-P) and 2.2-2.8(m,2H,CH_2-S).

Dicyclohexylamine (0.36g, 2mmole) was added to the above gum (0.13g, 0.9mmol) dissolved in 4mL of methanol and the resulting solution was concentrated to a small volume. Addition of 15mL of acetone precipitated a white solid, which was recrystallized from acetone/methanol mixture to

furnish the dicyclohexylamine salt of 2-(phosphono)ethyl thiol as a white crystalline solid, 0.45g (82% yield), mp 205-9°C, R_f=0.55 in 0.25M lithium chloride (iodine vapour) and DTNB spray (PEI Cellulose plates). NMR(D_2O): δ0.5-1.7(m,44H,cyclohexyl protons) and 1.8-2.8(m,4H,P-CH_2-CH_2-S).

2.3.6. O-PHOSPHO-L-MALATE

The synthesis of O-phospho-L-malate was first attempted using the free acid form of L-malic acid. The secondary hydroxyl was found to be unreactive to following phosphorylation reagents (using standard literature conditions for the phosphorylation of alcohols): dibenzylphosphorchloridate (Atherton et al., 1948), diphenylphosphorochloridate (Thorsett et al., 1982), phosphorous oxychloride (Nomura et al., 1969), pyrophosphorylchloride (Barrio et al., 1978), 2-cyanoethyl phosphate (Tenner, 1961; Wehrli et al., 1965), and the tri-n-butylamine salts of phosphate and pyrophosphate (N,N'-dicyclohexylcarbodiimide as coupling reagent) (Myers et al., 1965). To make the hydroxyl more reactive, esterification of the carboxyl groups of L-malic acid was accomplished with benzyl alcohol by the method of Smith et al. (1965). The crude product was purified by silica column chromatography using hexanes:ethyl acetate (80:20) as eluent. The isolated white solid (97% overall yield) was identified as dibenzyl-L-malate by NMR. NMR($CDCl_3$):

δ2.8(d,J=5Hz,2H,CH_2), 3.2(m,1H,OH), 4.4(t,J=5Hz,1H,CH), 5.1(s,2H,$ArCH_2$), 5.2(s,2H,$ArCH_2$) and 7.3(s,10H,ArH).

The hydroxyl group was now found to be sufficiently active to be phosphorylated by many reagents. However, TLC analysis showed that the phosphorylated product spontaneously eliminated phosphate to yield a mixture of the cis and trans elimination product, dibenzyl maleate and dibenzyl fumarate. This elimination is similar to the spontaneous elimination of phosphate observed within the active site of mammalian pyruvate kinase when the nitro analogue of L-malate, 3-nitrolactate is phosphorylated (Porter et al., 1983). The same elimination products were identified when dibenzyl-L-malate was reacted with methanesulfonylchloride. Further synthesis with dibenzyl-L-malate were abandoned since promising results were available with other inhibitors involving less synthetic difficulty.

2.3.7. 3-NITRO-2-CYANOMETHYL ACRYLATE

The synthetic scheme attempted can be seen in Fig. 2.1. Solid (dry) sodium ethoxide (3.4g, 50mmole) was added slowly to a cooled (4^{o}C) very dry solution of methylcyanide (2g, 50mmole) and diethyl oxalate (6.95mL, 50mmole) in 50mL of diethyl ether. The solution was allowed to warm to room temperature. The yellow precipitate that appeared was filtered off 12h later and dried. The solid obtained

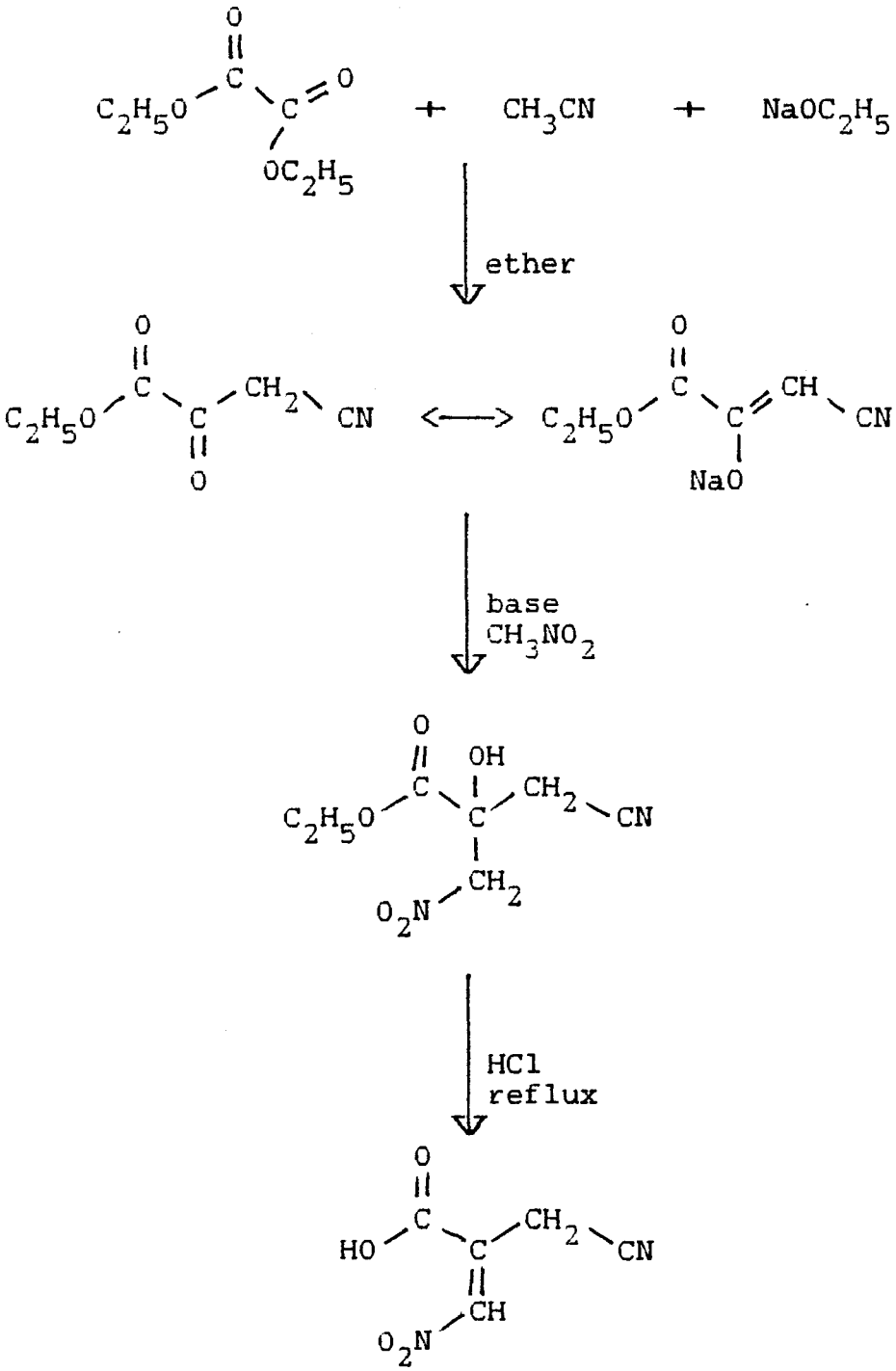


FIGURE 2.1 -- Strategy for the synthesis of 3-nitro-2-cyanomethyl acrylate.

(9.93g) was found to be impure by TLC analysis. The solid was dissolved in 1M phosphoric acid and extensively extracted with chloroform. The chloroform was removed and the solid dried (7.6g, 93% yield). This solid showed one spot on TLC (iodine detection) and identified by NMR as ethyl cyanopyruvate. NMR($CDCl_3$): δ1.4(t,J=7Hz,3H,$-CH_3$), 4.4(q,J=7Hz,2H,$-CH_2-CH_3$) and 5.5(s,2H,$-CH_2CN$).

The addition of nitromethane to sodium ethyl cyanopyruvate was attempted using many conditions. The bases sodium bydroxide, calcium hydroxide and sodium potassium carbonate were tried at various strengths; water, nitromethane and methonal were tried as solvents, both alone and in various combinations; various reaction temperatures from -10^{o} to 50^{o}C were tried (Blicke et al., 1952; Nightingale & Janes, 1944; Hass & Bourland, 1947; Sprang & Degering, 1942; and Worrall, 1929). In no case was a simple product mixture realized. By following the reactions with TLC, the dissappearance of starting materials was accompanied by the appearance of a multitude of unidentified spots (>5). It appears that the methylene group was too acidic for this reaction to proceed as desired. A new synthetic scheme needs to be designed before this compound can be synthesized.

3. RESULTS

3.1. INHIBITOR STUDIES

The enzyme assay using [^{14}C]-carbamoyl phosphate was used for all inhibitor studies reported. This assay displayed the sensitivity and precision necessary for valid comparisons to be made between the effects of different substituents. The variances listed for the K_i determinations are the observed ranges for several separate measurements.

Initially, consideration was given to the design of transition state analogues that would interact most favourably at all four regions of the catalytic site (see Fig. 1.7). Thus, the analogues would contain an aspartate moiety (to interact at the dicarboxylate and amino regions) and a phosphate or phosphonate group (to interact at the phosphate region). It was felt that the transition state had a tetrahedral anionic group within the carbamoyl region (Fig. 1.10). To imitate this "natural" transition state, a search was undertaken for functional groups that, when placed between the aspartate and phosphate groups, would be anionic and tetrahedral. A phosphate group at this position was deemed to be similar enough in geometry and ionic character to be worthy of synthesis.

No simple one-step condensation of L-aspartate and pyrophosphate to form N-pyrophosphoryl-L-aspartate was

successful, so a step-wise addition of phosphate groups to L-aspartate was undertaken. The addition of the first phosphate group was only successful when protecting groups and organic solvents were employed. The deprotected product, N-phosphoryl-L-aspartate, was observed to be very succeptible to hydrolysis in aqueous solutions. For this reason, the addition of another phosphate group was not attempted.

N-Phosphoryl-L-aspartate was tested for its inhibitory power on c_3 (TABLE I). It was found to inhibit competitively against carbamoyl phosphate with an extremely low binding constant (K_i=50μM). This was especially noteworthy when one considered that N-phosphoryl-L-aspartate lacked the second phosphate group which was thought to be a requirement for tight binding. Since no other tetrahedral anionic groups that could be placed between L-aspartate and phosphate was apparent, a detailed study of the qualities of the group placed within the carbamoyl site that would maximize the binding affinity was performed. Since the carbamoyl region is too small to interact strongly with ligands by itself, compounds, both those commercially available and those easily synthesized, which bind primarily to the adjacent regions were tested. The use of compounds which bound primarily to regions adjacent to the carbamoyl region allowed predictions to be made as to their probable mode of binding.

FOOTNOTES TO TABLE 1

[a] All kinetic studies were performed at pH 7.5 and 25°C with 15mM L-aspartate and 10-50μM carbamoyl phosphate.

[b] All N-substituted analogues were of the L-configuration except for N-carbamoyl-DL-aspartate.

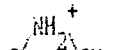
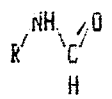
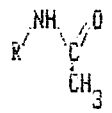
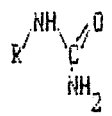
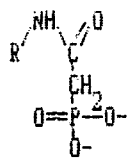
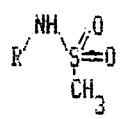
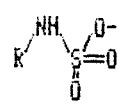
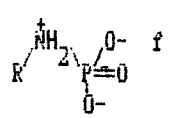
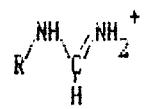
[c] The nature of inhibition was determined to be competitive (C), uncompetitive (UC) or noncompetitive (NC) against carbamoyl phosphate.

[d] Literature values under similar conditions are: succinate 3.5mM and L-aspartate 20mM (Porter et al., 1969); N-carbamoyl-L-aspartate 1.5mM (Heyde et al., 1973); PALA ~0.000027mM at pH 7.0 (Collins & Stark, 1971).

[e] A noncompetitive pattern for this inhibitor has been reported by Porter et al. (1969) and Heyde et al. (1973). However, our results clearly show competitive inhibition (Fig. 3.1). We can find no explanation for the discrepancy apart from possible differences due to experimental conditions.

[f] This derivative is depicted as the zwitterion on the basis of previous work (Benkovic & Sampson, 1971).

TABLE I
Inhibition of ATCase by L-aspartate derivatives[a]

Compound[b] [R=-CH(COO$^-$)CH_2COO$^-$]	K_i (mM)	Inhibition[c] Pattern
	2.2±.03[d]	UC
	2.8±.4[d] (K_m)	-
	~40	NC
	7.4±.2	C
	6.9±.5	C
	1.9±.3[d]	C[e]
	0.00000050±00000007[d]	C
	9.1±.1	C
	0.49±.10	C
 [f]	0.05±.01	C
	~40	C

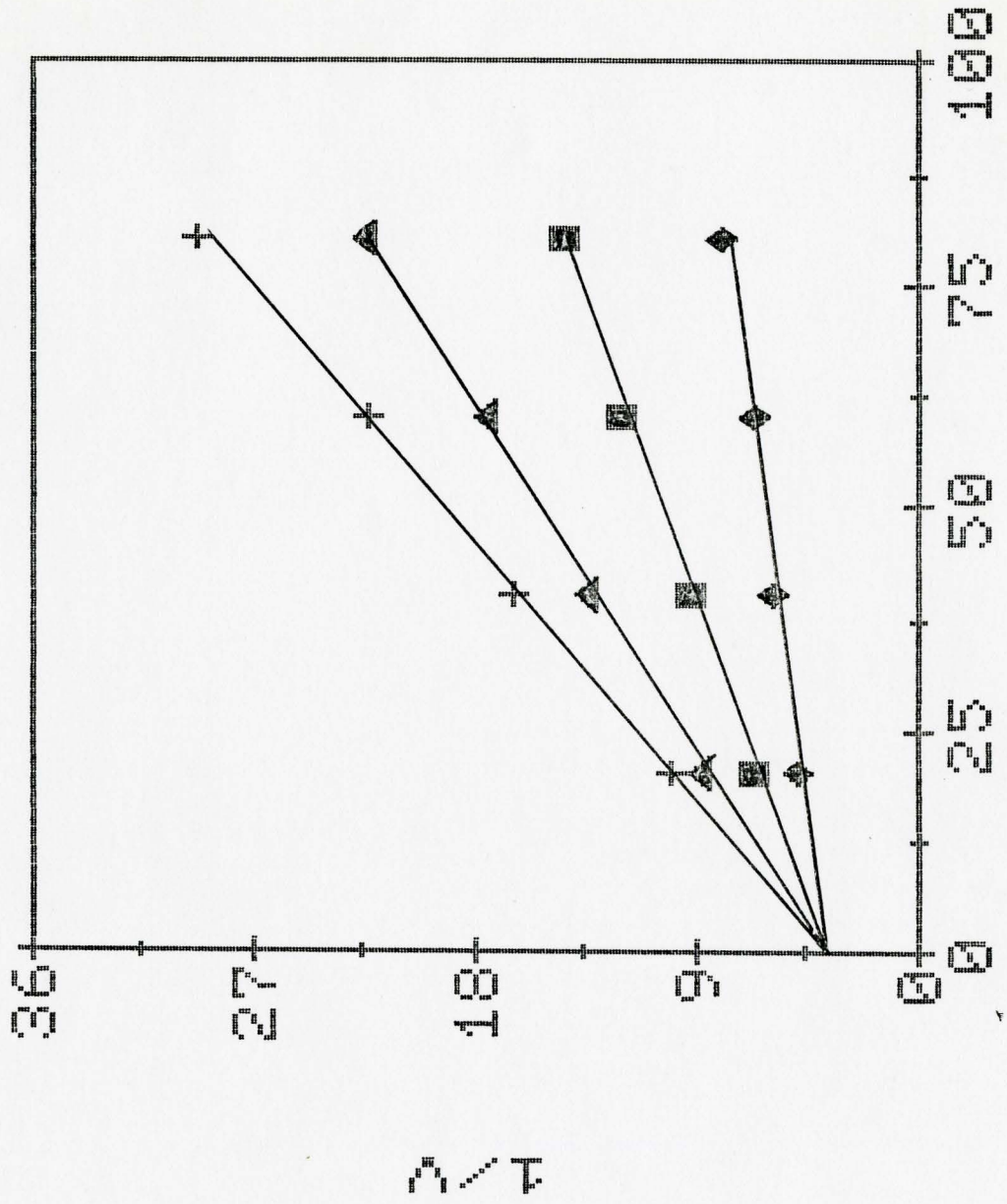
3.1.1. INHIBITION BY L-ASPARTATE DERIVATIVES

L-Aspartate derivatives were the first series of compounds to be investigated. When the amino group of L-aspartate was derivatized, the resulting compounds inhibited the enzyme in a competitive manner versus carbamoyl phosphate. The introduction of a methyl group resulted in a weak ligand (TABLE I). However, when the substituent contained a carbonyl group substantial improvement in binding occured in spite of the increase in bulk. The presence of an extra amido group in N-carbamoyl-DL-aspartate (Fig. 3.1) further enhanced the affinity by a significant (3.5 fold) factor presumably because of the additional hydrogen-bonding capacity. Extremely tight binding was observed when a phosphonoacetyl group was added to L-aspartate, PALA (K_i=0.50nM). This extremely tight affinity, 40 fold better than the literature value (Collins & Stark, 1971), may have been partially due to the assay design. The one-half hour incubation of enzyme plus inhibitor prior to the introduction of carbamoyl phosphate could have allowed the enzyme to be saturated with PALA. The slow dissociation of this complex in the presence of carbamoyl phosphate would then lead to a significant overestimation of its binding constant.

N-Methanesulfonyl-L-aspartate was synthesized to test the possibility that catalysis is facilitated by stabilization of a tetrahedral transition-state (TABLE I).

LEGEND TO FIGURE 3.1

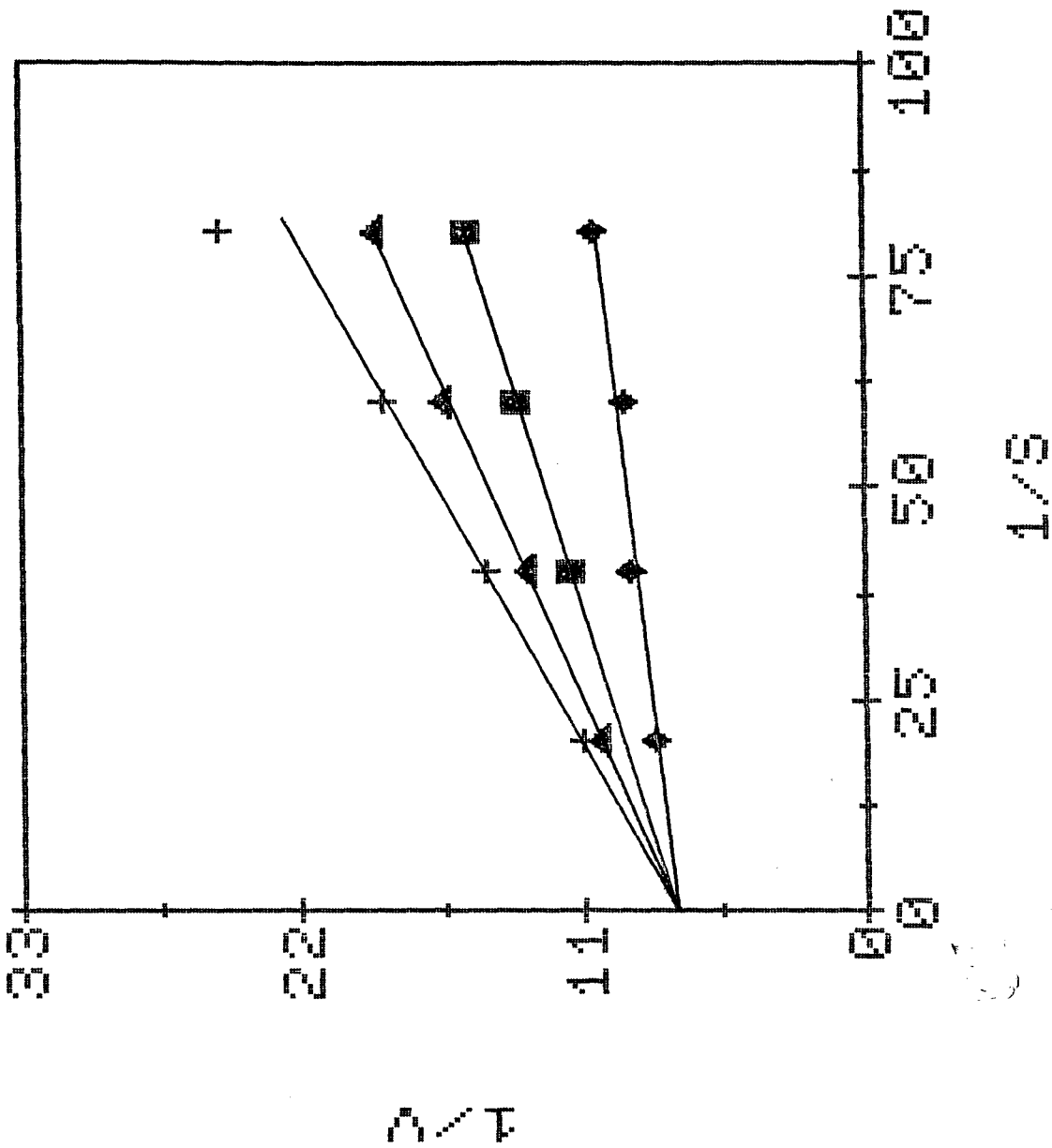
Inhibition pattern of N-carbamoyl-DL-aspartate. The inhibitor concentrations were: (◆), 0mM; (■), 4mM; (▲), 8mM; and (+), 12mM. The reaction velocities (V) are expressed as nmoles per minute per mg of catalytic subunit. S represents the concentration of the variable substrate (carbamoyl phosphate) in mM units. The concentration of aspartate was kept constant at 15mM.



3.1 -- Inhibition by N-carbamoyl-DL-aspartate

LEGEND TO FIGURE 3.2

Inhibition pattern of N-sulfo-L-aspartate. The inhibitor concentrations were: (◆), 0mM; (■), 0.55mM; (▲), 1.10mM; and (+), 1.65mM. The reaction velocities (V) are expressed as nmoles per minute per mg of catalytic subunit. S represents the concentration of the variable substrate (carbamoyl phosphate) in mM units. The concentration of aspartate was kept constant at 15mM.



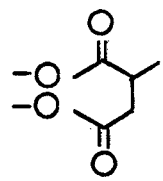
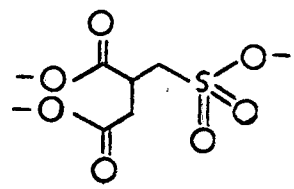
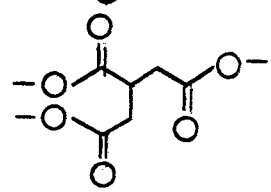
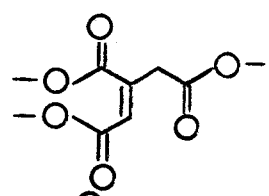
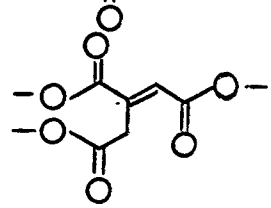
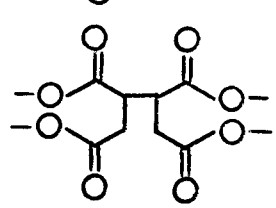
3.2 -- Inhibition by N-sulfo-L-aspartate

Surprisingly, this compound did not prove to be superior to its trigonal counter-part, N-acetyl-L-aspartate. Both N-sulfo-L-aspartate (Fig. 3.2) and N-phosphoryl-L-aspartate (synthesized earlier) were found to be substantially better inhibitors than the corresponding neutral derivative. The existance of ionic interactions is further supported by the poor binding of N-formimino-L-aspartate, which suggests repulsion of the positive charged group. Since the instability of N-carbamic-L-aspartate ($R-NH-COO^-$) precluded its study as an inhibitor, the study of methylsuccinic acid derivatives was undertaken to examine the stereochemical aspects of these ionic interactions (Dennis et al., 1985).

3.1.2. INHIBITION BY METHYLSUCCINATE DERIVATIVES

Methylsuccinate, like L-aspartate (TABLE II) has previously been reported to be a much weaker ligand than succinate (Davies et al., 1970). The introduction of a negatively charged sulfo-group onto methylsuccinate resulted in a dramatic increase in affinity, similar to that observed with the N-sulfo derivative of L-aspartate. The inhibition was also competitive against carbamoyl phosphate (Fig. 3.3). Replacement of the tetrahedral sulfo-moiety by a trigonal carboxyl group in tricarballylate made only a small difference to the binding. The two dehydro-derivatives of tricarballylate (cis- and trans-aconitate) were found to be

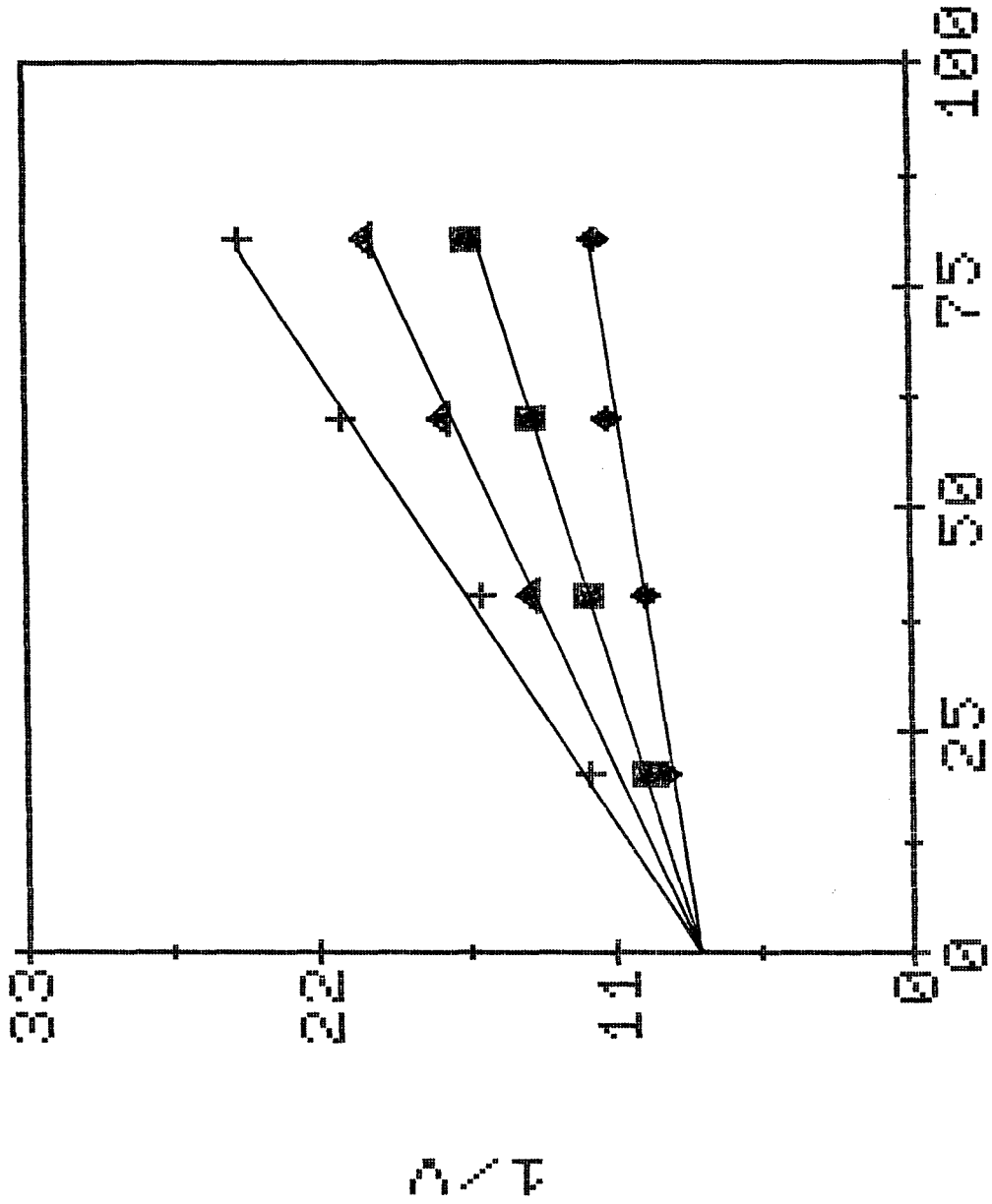
TABLE II
Inhibition of ATCase by methylsuccinate derivatives[a]

Compound	K_i (mM)
	16±2
	2.9±.1
	1.2±.1
	0.48±.09
	0.61±.10
	0.3±.1

[a] For conditions see TABLE I. All compounds tested here show competitive inhibition against carbamoyl phosphate.

LEGEND TO FIGURE 3.3

Inhibition pattern of DL-2-sulfomethylsuccinate. The inhibitor concentrations were: (◆), 0mM; (■), 3.5mM; (▲), 7.0mM; and (+), 10.5mM. The reaction velocities (V) are expressed as nmoles per minute per mg of catalytic subunit. S represents the concentration of the variable substrate (carbamoyl phosphate) in mM units. The concentration of aspartate was kept constant at 15mM.



3.3 -- Inhibition by DL-2-sulfomethylsuccinate

even better inhibitors (Dennis et al., 1985). Addition of a further carboxylate to tricarballylate, 1,2,3,4-butanetetracarboxylate, resulted in a significant increase in affinity.

3.1.3. INHIBITORS SELECTED TO DEFINE MODE OF BINDING

To determine the mode of binding of the L-aspartate and methlysuccinate derivatives, several other inhibitors were tested. N-Methanesulfonyl-D-aspartate and N-sulfo-D-aspartate were synthesized and found to inhibit the enzyme as strongly as their L counterparts (Table III). N-Sulfo-L-glutamate was also synthesized and found to inhibit the enzyme nearly as strongly as N-sulfo-L-aspartate. It should be noted that the poor ligand L-glutamate inhibited the enzyme in a competitive manner. Threo $D_s(+)$ isocitrate was observed to have a binding constant better than tricarballylate, the DL-methylsuccinate derivative lacking the hydroxyl group.

3.1.4. INHIBITION BY PHOSPHONO DERIVATIVES

Numerous phosphate and phosphonate derivatives have been reported previously to act as strong inhibitors (Porter et al., 1969). In particular, the remarkably high affinity of pyrophosphate may be attributed to the binding of the second charged group. To confirm this

TABLE III

Inhibitors of ATCase selected to define mode of binding[a]

Compound	K_i (mM)
N-Methanesulfonyl-D-aspartate	9.7±.3
N-Sulfo-D-aspartate	0.87±.09
L-Glutamate	65±5[b]
N-Sulfo-L-glutamate	0.90±.10
Threo D_s(+) isocitrate	0.24±.02

[a] For conditions see TABLE I. All compounds tested here show competitive inhibition against carbamoyl phosphate.

[b] Literature value is 120mM (Davies et al., 1970).

possibility, the analogous compound, methylenediphosphonate was tested and found to inhibit the enzyme to approximately the same extent (TABLE IV). Phosphonoacetate by comparison, was a much weaker inhibitor, suggesting that the trigonal carboxyl group was not optimal for the specific ionic interaction. However, the shorter homologue phosphonoformate displayed an unexpectedly tight binding to the enzyme. The favourable interactions with compounds containing a second negatively charged moiety was also reflected in the greatly reduced affinity of phosphonate derivatives containing a positively charged amino group. In contrast, neutral moieties such as methoxy and mercaptoethyl groups had relatively little effect (Dennis et al., 1985).

3.1.5. INHIBITION BY L-MALATE DERIVATIVES

The synthesis of O-malate derivatives was attempted since it was thought that the hydroxyl group allowed relatively easy derivatization. Unfortunately, derivatives such as O-phosphoryldibenzyl-L-malate and O-methanesulfonyl-dibenzyl-L-malate underwent elimination to give dibenzylfumarate and dibenzylmaleate during synthesis. Again, a compound that interacted at all subsites of the active site and resembled the postulated transition state (O-pyrophosphoryl-L-malate) eluded synthesis.

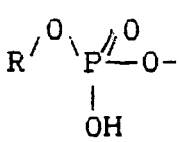
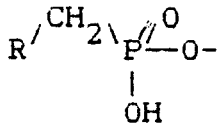
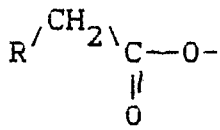
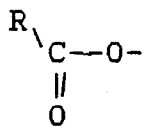
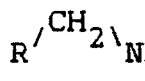
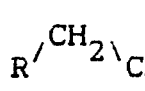
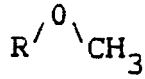
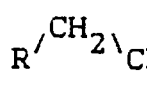
FOOTNOTES TO TABLE IV

[a] For conditions see TABLE I. All compounds tested here show competitive inhibition against carbamoyl phosphate.

[b] Literature values range from 0.6mM (Heyde & Morrison, 1973) to 1.45mM (Porter *et al.*, 1969).

[c] The constants reported by Porter *et al.* (1969) for these inhibitors are approximately twice the value given here.

TABLE IV
Inhibition of ATCase by phosphorus-containing inhibitors[a]

Compound ($R = -PO_3^{2-}$)	K_i (mM)
	0.36±.05[b]
	0.051±.002[c]
	0.037±.003
	0.15±.02[c]
	0.022±.004
	>40
	>40
	0.53±.01[c]
	0.91±.05

3.1.6. INHIBITION BY CYCLIC COMPOUNDS

At this point it was decided that no stable aliphatic inhibitor could be designed to take advantage of the ionic interactions within the carbamoyl site and still interact at all the other regions. To overcome these stability problems, the possibility of using cyclic compounds as inhibitors for the catalytic subunit was investigated (TABLE V). All the compounds tested inhibited the enzyme in a competitive manner. The compound 3,4-furandicarboxylic acid and phthalic acid were found to inhibit the enzyme with binding constants slightly better than succinate. 1,2,4-Benzenetricarboxylic acid bound approximately 7.5 fold better than phthalic acid, indicating a favourable interaction with the additional carboxyl. By substituting a tetrahedral anionic group for this additional carboxyl, 4-sulfophthalic acid, the affinity for the enzyme was not greatly affected. Several ring compounds containing four carboxyl groups were tested and found to be potent inhibitors. The best, 1,2,4,5-benzenetetracarboxylic acid , has a K_i of 50μM (Fig. 3.4). The inhibitor 1,2,3,4-cyclopentanetetracarboxylic acid (all cis) was found to be a 3 times poorer inhibitor than the comparable benzene analogue. The inhibitor tetrahydrofuran 2,3,4,5-tetracarboxylate, although composed of a mixture of diastereoisomers, inhibited the enzyme better than the cyclopentane (all cis) analogue and comparable to the

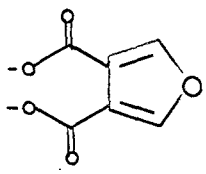
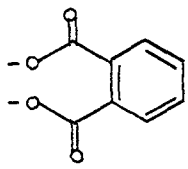
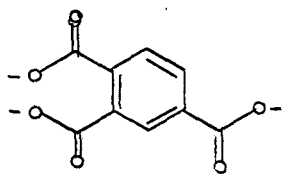
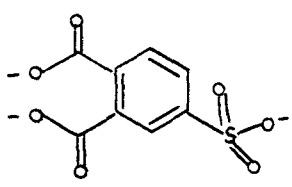
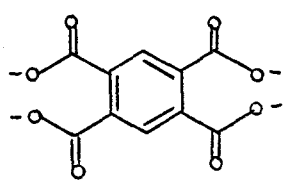
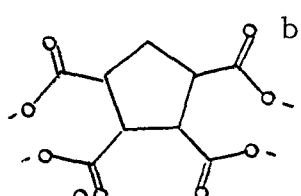
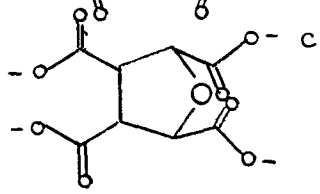
FOOTNOTES TO TABLE V

[a] For conditions see TABLE I. All compounds tested here show competitive inhibition against carbamoyl phosphate.

[b] This compound was all cis.

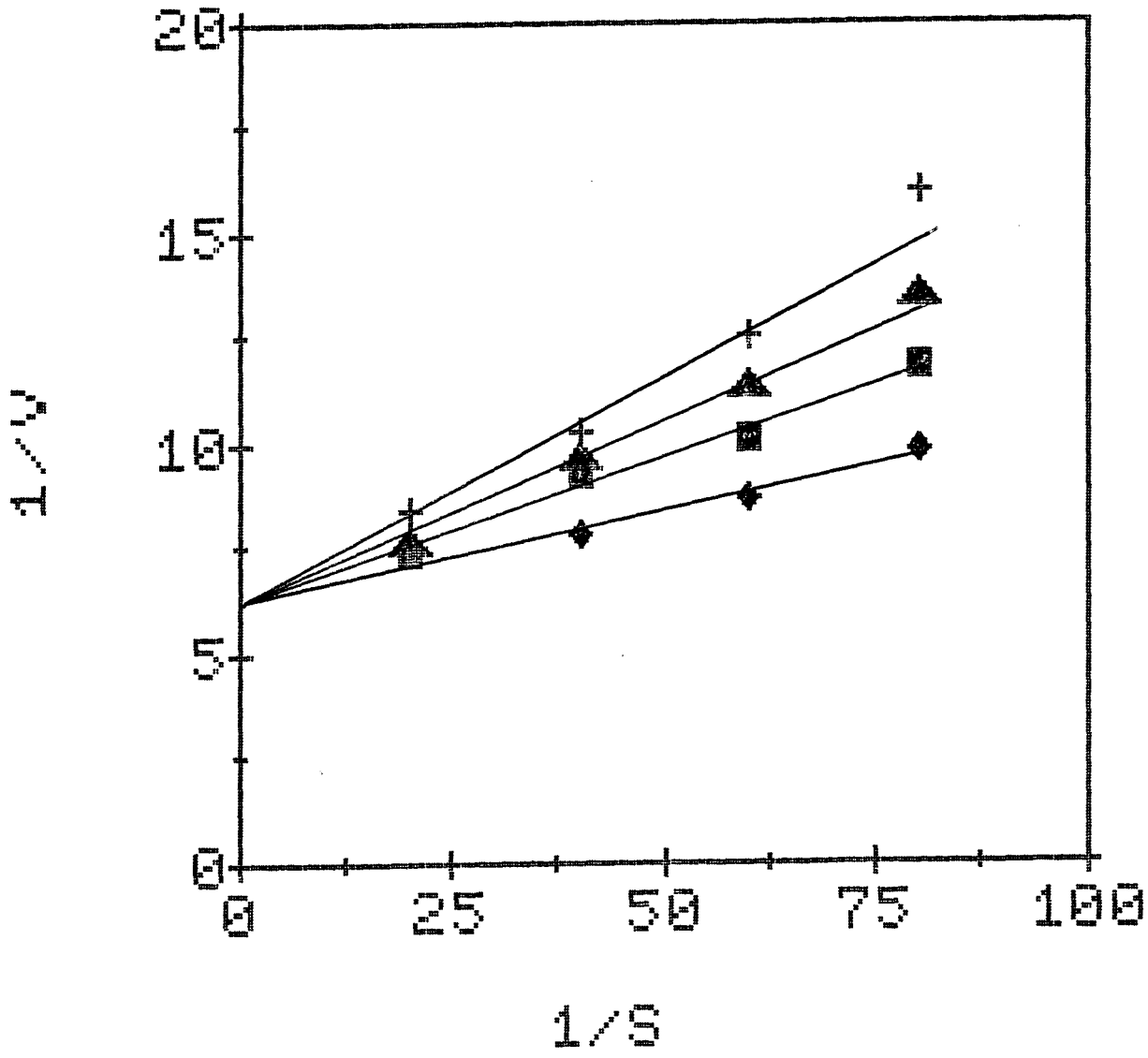
[c] This compound was composed of a mixture of diastereoisomers.

TABLE V
Inhibition of ATCase by cyclic compounds[a]

Compound	K_i (mM)
	1.5±.2
	1.2±.1
	0.16±.03
	0.14±.02
	0.050±.001
 [b]	0.15±.02
 [c]	0.060±.002

LEGEND TO FIGURE 3.4

Inhibition pattern of 1,2,4,5-benzenetetra-carboxylate. The inhibitor concentrations were: (◆), 0μM; (■), 30μM; (▲), 60μM; and (+), 90μM. The reaction velocities (V) are expressed as nmoles per minute per mg of catalytic subunit. S represents the concentration of the variable substrate (carbamoyl phosphate) in mM units. The concentration of aspartate was kept constant at 15mM.



3.4 -- Inhibition by 1,2,4,5-benzenetetracarboxylate

benzenetetracarboxylic acid inhibitor.

3.1.7. SUICIDE INHIBITOR

The postulated suicide inhibitor, 3-nitro-2-cyanomethyl acrylic acid (Fig. 1.11), has yet to be synthesized. The attempted synthetic pathway (Fig. 2.1) was unsuccessful. It remains to be seen if this compound could be a very specific and potent inhibitor of ATCase.

4. DISCUSSION

The inhibitors used in this study were tested against carbamoyl phosphate. Enzyme assays in which carbamoyl phosphate is the varied substrate are made very difficult because of the 400 fold difference in K_m's for the two substrates (K_m(aspartate)=2.8mM; K_m(carbamoyl phosphate)=8μM). The use of a radioactive assay was deemed necessary to detect the less than 2nmoles of product each assay would produce.

The initial assay using [^{14}C]-L-aspartate has been successfully used to study inhibitors competitive against L-aspartate (adapted from Porter et al. (1969)). To use this assay for the study of inhibitors competitive against carbamoyl phosphate, the concentration of L-aspartate was made rate limiting by reducing it below the apparent K_m for L-aspartate. The detection of a low level of conversion of L-aspartate to N-carbamoyl-L-aspartate was still difficult. To prevent kinetic changes during the time course of the experiment, less than 20% of the available carbamoyl phosphate was allowed to react. This often resulted in less than 2% of the available L-aspartate being converted to product. Since labelled contaminants could not be entirely removed from the [^{14}C]-L-aspartate, the assays demonstrated unacceptably high background counts.

The recent availability of [^{14}C]-carbamoyl phosphate

made it possible to design an assay using this label. Modification of the assay developed by Swyryd et al. (1974) yielded a sensitive assay with the desired low background suitable for the study of the catalytic subunit of ATCase.

4.1. BINDING MODE OF INHIBITORS

The use of enzyme kinetics to study the environment of the active site of an enzyme has several inherent difficulties. The most relevant drawback is the uncertainty about the mode of binding of the inhibitor. If the inhibitor is based upon a substrate, it is often assumed that that part of the inhibitor that resembles the substrate binds in the same manner as the substrate (from hereon termed the normal binding mode). The change in affinity which is observed for each inhibitor is then attributed to the additional/different functional groups. Inhibitors may however bind in an abnormal manner leading to a misinterpretation of the data.

The enzyme ATCase has two relatively small substrates. The majority of previous inhibitor studies used substrate analogues that were designed to bind in one or the other substrate pockets. By testing the competitiveness of the inhibitor, previous researchers were able to define the site of binding as that to which the normal substrate bound

(see Section 1.3.4.). It was felt that the inhibitors bound in a manner very similar to the natural substrate. Also, the carbamoyl phosphate binding site, by virtue of being saturated with the normal substrate in previous assays (K_m~8μM, normal carbamoyl phosphate concentrations used were greater than 1mM), was unavailable for the abnormal modes of binding (primary interaction in the carbamoyl phosphate site) of dicarboxylate analogues. The bisubstrate analogues, PALA, DIKEP, and DIHOP were thought to bind in the same manner as the substrates according to several lines of evidence (inhibition pattern, UV difference spectroscopy and activation of native enzyme (Collins & Stark, 1971)).

This study, however, includes many compounds based on succinate or L-aspartate (TABLES I, II, and III) with groups that were designed to extend into the carbamoyl region (see Fig. 1.7). Abnormal modes of binding can not be ruled out simply by observing that the inhibitors were competitive against carbamoyl phosphate. For an inhibitor to be competitive against carbamoyl phosphate, it is only necessary that the presence of the inhibitor within the acitive site of ATCase prevents the binding of carbamoyl phosphate. The inhibitor may express this competitiveness by physically or electrostatically blocking the carbamoyl and/or phosphate region. Also, although a portion of the inhibitor's structure resembles the natural substrate L-aspartate, it may not be assumed that it will bind in the

normal manner within the dicarboxylate region. This is especially true when anionic groups are added to L-aspartate since the tight binding of phosphate indicates that the phosphate region has a high affinity for anionic groups.

Previous work by other researchers (Porter et al., 1969; Davies et al., 1970) has defined a set of structural conditions necessary for normal binding within the dicarboxylate region (see Section 1.3.4.(a)). By applying these rules the following inhibitors, or a subgroup of them in the case of a DL mixture, cannot bind in the normal mode (whereby the inhibitor fully occupies the dicarboxylate region with substituents reaching into the carbamoyl region): N-carbamoyl-D-aspartate; D-methylsuccinate; D-sulfomethylsuccinate; D-tricarballylate; threo $D_s(+)$ isocitrate; N-methanesulfonyl-D-aspartate; and N-sulfo-D-aspartate. Inhibitors having substituents on a trigonal α-carbon have not been previously tested so no conclusions can be drawn regarding cis- or trans-aconitate.

The tight binding of threo $D_s(+)$ isocitrate relative to tricarballylate indicates that abnormal binding modes must be available to the inhibitors. It is impossible for this compound (because of its D configuration) to bind to the dicarboxylate region and the carbamoyl region at the same time according to the constraints discovered by Davies et al. (1970). Also, the threo dicarboxylate compounds

have been shown to be unable to bind efficiently to the dicarboxylate region (Davies et al., 1970). Therefore, one of the carboxyls must interact within the phosphate region. If it is possible for the dicarboxylate base inhibitors to interact favourably within the phosphate region, then it cannot be assumed that the other analogues bind primarily in the dicarboxylate region and reach into the carbamoyl region.

The most glaring inconsistancy within the data concerns the affinity of the N-methanesulfonyl- and N-sulfo-aspartates. The difference in affinity between the D and L compounds was found to be very slight, almost insignificant. If one assumes that the D compound's dicarboxylate moiety is anchored within the dicarboxylate region, then the additional group must reach into the phosphate region (not the carbamoyl region). For this mode of binding to be plausible, the phosphate and carbamoyl regions must have identical affinities towards the methanesulfonyl and sulfo groups. This would be an extremely unlikely occurrence. More likely, both pairs of D and L compounds bind in a similar manner, the stereochemistry about the α-carbon being unimportant. This abnormal mode of binding may involve a primary interaction within the phosphate binding site, since this very small region is known to hold anions quite tightly. The remainder of the inhibitor would then reach out to interact wherever possible. The possibility of this

abnormal mode of binding may have been enhanced during this study by the use of an enzyme assay where the substrate concentrations were varied about their respective K_m's (previous assays used saturating amounts of carbamoyl phosphate).

The unusual binding mode described above would explain the unusual observation that N-carbamoyl-DL-aspartate is a competitive inhibitor against carbamoyl phosphate (TABLE I). Several previous workers (Porter et al., 1969; Heyde et al., 1973) have observed N-carbamoyl-L-aspartate to be a non-competitive inhibitor against carbamoyl phosphate.

The mode of binding of the phosphate/phosphonate containing inhibitors (TABLE IV) is not known. Since the phosphate region shows the greatest affinity for anions, it is assumed that they bind primarily to this region. Where the other groups bind is not absolutely known, but it is assumed that at least the inhibitors containing other anionic groups reach into the carbamoyl region since pyrophosphate has been shown to bind in this manner (Honzatko & Lipscomb, 1982).

The cyclic compounds present another difficulty. Only the binding mode of the compound 1,2,4,5-benzenetetracarboxylate is known -- all carboxyls are necessary for tight binding and, therefore, the dicarboxylate, phosphate and carbamoyl regions appear to be

occupied. The kinetic methods employed in this study do not permit determination of the regions left unoccupied by the other cyclic compounds.

4.2. IONIC INTERACTIONS WITHIN THE CARBAMOYL SITE

Previous researchers have documented the preference for a 4-carbon cis dicarboxylate moiety (dicarboxylate region) and a phosphate (phosphate region) (see Section 1.3.4.). This research indicates the presence of an additional anionic binding site within the carbamoyl region.

The addition of another carboxylate to 1,2,4-benzenetricarboxylate or to tricarballylate (Fig. 4.1) caused a modest increase in affinity (3 to 4 fold). The two compounds would have sufficient anions to interact with the previously known anion binding sites. Therefore, the increase in affinity due to the fourth carboxyl can be attributed to an uncharacterized anion binding site. This fourth site most plausibly occurs within the carbamoyl region.

Further support for the assignment of the putative interaction to the carbamoyl region is provided by the behavior of phosphonate derivatives with positively charged substituents. In these cases, there is no reason for the side chains to prefer the dicarboxylate region since they carry a charge opposite to that required for binding there. Both the aminomethyl and the aminoethyl groups are less

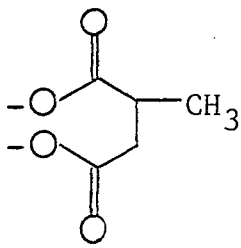
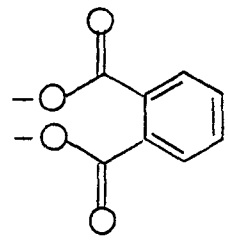
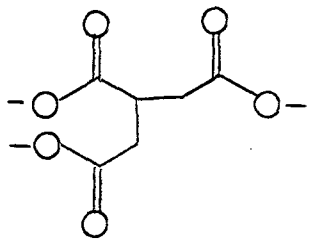
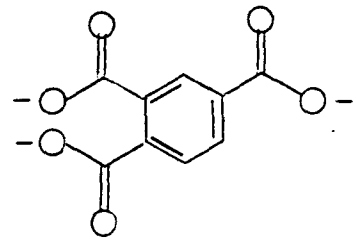
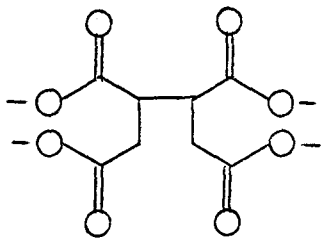
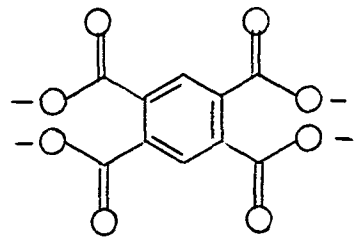
Inhibitor	K_i (mM)	Inhibitor	K_i (mM)
	16		1.2
	1.2		0.16
	0.30		0.050

FIGURE 4.1 -- Dependence of inhibitor affinity upon the number of carboxylate groups.

bulky than carbamoyl and would not be expected to encounter steric hindrance. Thus the extremely low affinity of these compounds must be attributed to electrostatic repulsion in the carbamoyl region. Also, pyrophosphate has been shown to bind to both the phosphate and carbamoyl regions (Honzatko & Lipscomb, 1982). The evidence therefore indicates the presence of a positively charged side chain in the carbamoyl region of the enzyme.

To maximize the available ionic interactions, precise placement of the anionic group within the carbamoyl region was found to be necessary. This is most clearly demonstrated by the relative affinities of phosphonoacetate and phosphonoformate (TABLE IV). It may be assumed that phosphonoformate placed its carboxylate closer to the positive group on the enzyme, increasing its affinity relative to the longer homologue, phosphonoacetate.

The exact role of this charged side-chain within the carbamoyl region is unknown. However, two points suggest that it may play an important role in catalysis. First, the carbamoyl group of the substrate and product are neutral groups, unable to be bound very tightly by a cation. Second, the carbamoyl region is the site of chemical catalysis so any group whose presence cannot be efficiently utilized by the substrate/product may be involved in promoting the reaction. This contribution to the chemical reaction could take several forms. The charged side-chain

may be the acid catalyst which directly donates a proton to the carbamoyl oxygen in the transition state (see section 1.3.6.3.(c)), such a process being consistent with the ^{13}C-NMR data (Roberts et al., 1976). The pK_a of such a proton donor would necessarily be near the pH of the reaction. To create such a situation, the charged side-chain could interact with an ionizable side-chain such as that of tyrosine to lower its pK_a. Another possible role for this charged group would be for it to stabilize the negative charge that develops as the lone pair electrons on the aspartate nitrogen attack to form a tetrahedral intermediate. A similar electrostatic stabilization of the transition state is considered the major factor in promoting the reaction catalysed by lysozyme (Warshel & Levitt, 1976).

The identity of the charged group within the carbamoyl region cannot be definitively identified by the results of this study. However, by combining the observations made during this kinetic study with the substantial body of data already accumulated, a substantial case may be built as to its identity. Chemical modification and X-ray crystallographic studies (see section 1.3.5.) have shown the presence of arginines in the active site. The location of Arg54 and Arg105 suggest that they may be involved in phosphate binding while Arg167 is a possible candidate for interactions at the carbamoyl region. Several lysines have also been identified as essential residues.

The most discussed lysine, Lys164, is in a position to interact with Tyr165 (lowering its pK_a), but these residues are too far away to have an effect on the carbamoyl region (Honzatko & Lipscomb, 1982). The group most likely involved in the binding of the above inhibitors is His134 (see section 1.3.5.(c)). In the difference map obtained by X-ray diffraction of the enzyme-pyrophosphate complex (Honzatko & Lipscomb, 1982), the imidazole ring of this residue is in van der Waals' contact with the second phosphate group. Since the charge of a protonated histidine side chain may be delocalized between the two imidazole nitrogens, significant interactions may occur with inhibitor functional groups at different positions. This variation in the location of the charge may explain some of the unexpected results presented here (eg. the tight binding of both pyrophosphate and phosphonoformate) (Dennis et al., 1985).

4.3. TETRAHEDRAL VERSUS TRIGONAL TRANSITION STATE

The nucleophilic attack of an amine (ie. aspartate) on carbamoyl phosphate is generally considered to proceed via a tetrahedral intermediate (Stark, 1971). An interesting question is whether the enzyme lowers the activation energy by geometric destabilization of the substrate relative to the transition state (see section 1.1.3.). One possibility is for the active site to be complimentary to the tetrahedral intermediate. In fact, it

has been suggested that substrate analogues with a tetrahedral configuration at the carbamoyl carbon might bind more tightly to aspartate transcarbamoylase (Jacobson & Stark, 1973).

The L-aspartate and methylsuccinate based inhibitors were designed to be anchored within the dicarboxylate region reaching into the carbamoyl region. It was hoped that the differences between these derivatives would yield information as to the preference of the carbamoyl region. Unfortunately, the unusual binding mode of many of these inhibitors prevented an exact assignment as to which group was interacting within the carbamoyl region.

The results using the phosphate/phosphonate based inhibitors are very limited. It appears that there is very little discrimination between tetrahedral and trigonal anions placed within the carbamoyl region. The greatest range is an increase in affinity of approximately four fold comparing methylenediphosphonate to phosphonoacetate (Table IV). However, the trigonal shorter homologue of phosphonoacetate, phosphonoformate, is bound slightly more tightly than methylenediphosphonate.

At this time it is impossible to draw firm conclusions concerning the geometric preference of the carbamoyl region. Either more phosphate/phosphonate inhibitors must be synthesized and tested or the L-aspartate/methylsuccinate based inhibitors must be somehow

"induced" to bind in the normal mode.

It is interesting to note that the tetrahedral alcohol (DIHOP) obtained by the reduction of DIKEP is also a very poor inhibitor compared to its parent compound (see section 1.3.4.(c)). One possible explanation is that the geometry of the transition state being stabilized lies somewhere between the trigonal form of the substate (or product) and the tetrahedral form of the intermediate. In any case, electrostatic interactions may be generally more effective than physical distortion as a catalytic mechanism since it does not require the enzyme active site to be extremely rigid (see section 1.1.3.) (Dennis et al., 1985).

4.4. RELATIVE ORIENTATION OF BINDING SUBSITES

The postulated catalytic mechanism of ATCase as depicted in Fig. 1.10 was not meant to suggest that the two substrates are held within the active site in the same plane. It is statistically unlikely that the two substrates would be held in that orientation considering the number of other possibilities. The use of cyclic reversible inhibitors, originally chosen to overcome stability problems, gave some insight into the relative orientation of the binding subsites.

It appears that inhibitors of ATCase based on planar

rings (the size of benzene or smaller) are not subjected to the same stereochemical constraints as are L- and D-configuration aliphatic dicarboxylate inhibitors (see section 4.1.). For this reason, it remains unknown to which sites the anionic groups of 3,4-furandicarboxylate, 1,2,4-benzenetricarboxylate and 4-sulfophthalate interact (TABLE V). Since it has been shown that all the carboxyls of 1,2,4,5-benzenetetracarboxylate are required for tight binding, the subsites for the binding of substrates within the active site must be oriented very closely to the same plane. The results also suggest that the phosphate binding site must be adjacent to the binding site of β-carboxylate within the dicarboxylate site (as depicted in Fig. 1.7). Considering the number of other feasible possibilities open to the evolving enzyme, this is a very surprising result.

4.5. DESIGN OF POSSIBLE TRANSITION STATE ANALOGUES

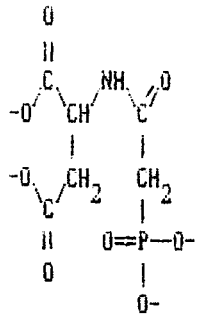
PALA remains the most tightly bound reversible inhibitor of the E. coli ATCase so far discovered (K_i~0.5nM). However, the results of this survey indicate that improvements can still be made. These changes would be directed at that part of the inhibitor that occupies the carbamoyl site. It is at this site that the chemical reaction occurs (transfer of the carbamoyl group) and therefore, it is here where the highest gains in affinity can be realized. The tight binding of PALA is due, not to

its resemblance to the transition state, but because it resembles both substrates and thus, has a large entropy contribution.

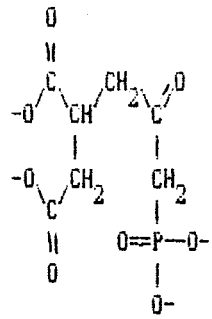
The existance of ionic interactions within the carbamoyl site suggests that the aliphatic compounds N-pyrophosphoryl-L-aspartate, O-pyrophosphoryl-L-malate and DL-2-pyrophosphorylmethylsuccinate (illustrated in Fig. 4.2) might have the highest affinity for the enzyme. The synthesis of all of the preceeding compounds were considered but abandoned at various stages due to encountered difficulties.

The synthesis of N-pyrophosphoryl-L-aspartate was halted after the addition of a single phosphate to L-aspartate. This compound, although binding very tightly to the enzyme, demonstrated that the combination of the required succinate moiety with a phosphoramidate linkage results in a very unstable compound. The instability of such phosphoramidate inhibitors has been noted by other groups (Thorset et al., 1982). Similarly, the synthesis of O-pyrophosphoryl-L-malate did not proceed very far due to its instability during synthesis.

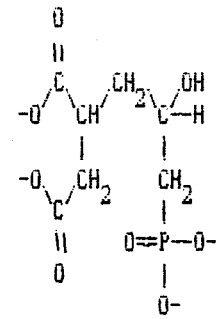
To take advantage of the ionic interaction within the carbamoyl site, cyclic compounds may be employed. The use of both saturated and unsaturated ring systems may allow the optimization of the available interactions by the correct placement of the functional groups. Also, the use



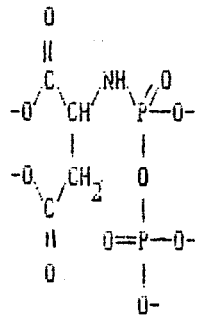
PALA



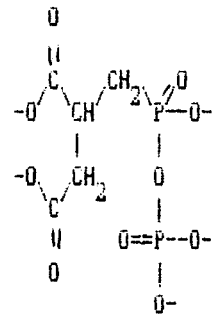
DIKEP



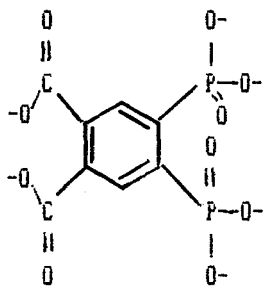
DIHOP



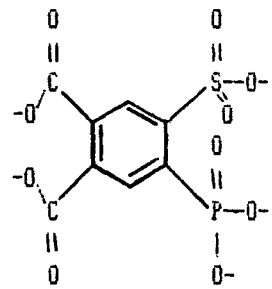
N-pyrophosphoryl-
L-aspartate



DL-2-pyrophosphono-
methylsuccinate



4,5-diphosphono-
phthalate



4-sulfo-5-phosphono-
phthalate

FIGURE 4.2 -- Structure of some of the best inhibitors, both those available and those proposed based on this work.

of cyclic compounds would eliminate many of the stability problems encountered with aliphatic inhibitors. Molecules such as 4,5-diphosphonophthalate and 4-sulfo-5-phosphonophthalate (illustrated in Fig. 4.2) may be the desired potent and specific inhibitors for ATCase. The interaction within the amino region should not be forgotten, inhibitors based on pyridine might give the desired results. Unfortunately, the synthesis of such cyclic compounds involves difficult synthetic techniques beyond the original scope of this research.

Another promising direction would be a study comparing inhibitor "length" with binding constant. The unusually tight binding of phosphonoformate compared to phosphonoacetate indicates that gains may be made by varying the separation between the groups interacting at the carbamoyl and phosphate regions. Further gains may be made if inhibitors with varying distances between the carbamoyl and dicarboxylate interacting moieties were designed.

4.6. DESIGN OF POSSIBLE IRREVERSIBLE INHIBITORS

Future irreversible inhibitors designed for E. coli ATCase should exploit the ionic interactions within the carbamoyl site. This could be as simple as small modifying anions such as periodate being used in conjunction with reversible inhibitors that occupy the carbamoyl region. Such studies could further delineate the environment of the

carbamoyl region. Another class of irreversible inhibitors might be designed containing phosphate or dicarboxylate moieties and incorporating a functional group for histidine (Dennis et al., 1985).

The synthesis of the postulated suicide inhibitor 3-nitro-2-cyanomethylacrylic acid was unsuccessful so the possibility that the enzyme would catalyse the series of proton transfers necessary to create the reactive keteneimine is unknown. To determine this, a different synthetic scheme must be planned so this compound can be isolated. The use of such a suicide inhibitor would not only be of pharmacological interest, but it would give the enzymologist a very useful tool for experiments involving isotope exchange or isotope labelling.

4.7. FUTURE USE OF INHIBITORS

It should not be forgotten that the enzyme from E. coli was chosen for study as a possible model for the mammalian enzyme. The previous, however limited studies on the mammalian ATCase have shown this enzyme to be part of a large multi-enzyme complex. Little work has been done to elucidate its catalytic mechanism, but it is thought to be very similar to that of the bacterial enzyme. This prediction has been based on the similar affinity of both enzymes for the inhibitor PALA. This one observation cannot

be used as proof for similar mechanisms. It would be of interest to determine if the mammalian enzyme has a similar ionic environment within the carbamoyl region as the bacterial enzyme. If this is true, it may be taken as further support that the E. coli enzyme can be considered a good model for the catalytic mechanism of the mammalian enzyme.

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