ACTIVE SITE STUDIES ON MICROSOMAL AMINOPEPTIDASE

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by

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ABSTRACT

The active site of porcine kidney microsomal aminopeptidase was investigated using single, multiple and EDTA inactivation kinetic studies. Good inhibitors invariably contained a zinc-coordinating group such as the mercapto moiety, which proved to be the best ligand for aminopeptidase. Due to the potency of β-mercaptoethylamine, derivatives of this compound were examined for aminopeptidase inhibition. (S)-2-amino-4-methyl-1-pentanethiol (L-leucinthiol) exhibited the largest potency and specificity towards aminopeptidase when compared against carboxypeptidase A and thermolysin, two similar zinc-peptidases.

The presence of a zinc-coordination subsite, two hydrophobic pocket subsites and a second amine-binding subsite (distinct from that responsible for substrate recognition) were discerned and the binding modes of amino acid hydroxamates and mercaptoamines compared using Yonetani-Theorell inhibition kinetics. Aminopeptidase does not show virtually any stereoselectivity between L- and D-leucine hydroxamate while greater than a 1,000-fold preference is seen for L-leucinthiol over the D isomer. Also, the amino group of mercaptoamines is crucial to the binding of these inhibitors whereas that of the hydroxamate compounds does not seem to contribute much to their binding. The differences in binding between hydroxamates and mercaptoamines are postulated to be a consequence of the product analogue nature of the former and transition state analogue character of the latter. L-leucine hydroxamate is proposed to bind in a backwards orientation

while the D isomer binds in the normal substrate-like position. Similarly, L-leucinthiol is proposed to bind in the same fashion as substrate.

Design of future inhibitors should endeavour to: (1) lower the pK_a of the α-amino group, (2) include an extended chain structure capable of binding to additional hydrophobic pockets, (3) incorporate a second amine moiety into the structure to interact with the second amine-binding subsite and (4) replace the mercapto group with a more potent zinc ligand such as the selenol group.

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ABBREVIATIONS

ACE	angiotensin converting enzyme
AUF	absorbance units full scale
collidine	2,4,6-trimethylpyridine
CPA	carboxypeptidase A
δ	NMR chemical shift, in ppm downfield from tetramethylsilane
DCCD	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
dithizone	diphenylthiocarbazone
DMF	dimethylformamide
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	(D,L)-dithiothreitol
EDTA	N,N,N',N'-ethylenediaminetetraacetic acid
FAPP	3-(2-furylacryloyl)-L-phenylalanine-L-phenylalanine monohydrate
h	Planck constant
Hz	hertz, cycles per second
IAA	isoamylamine
IR	infra-red
J	coupling constant (NMR)
k	Boltzmann constant
LeupNA	L-leucine p-nitroanilide hydrochloride
MAP	microsomal aminopeptidase

β-MEA	β-mercaptoethylamine hydrochloride
M_r	relative molecular mass
NMR	nuclear magnetic resonance
SDS	sodium dodecyl sulphate
T	absolute temperature
THL	thermolysin
TLC	thin layer chromatography
TNBS	trinitrobenzene sulphonic acid (or picrylsulphonic acid)
Tris	tris(hydroxymethyl)aminomethane

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1. INTRODUCTION

The first section of this chapter involves the relevance of enzymology to a concern of everyday life, illness. The classification of enzymes into groups and the rational, structure-function approach to the development of new drugs is illustrated using the angiotensin converting enzyme in the following section. The use of transition state theory and inhibition kinetics in the study of enzymes and design of inhibitors is examined in the next four sections. Finally, the last section deals with microsomal aminopeptidase, its proposed physiological role in modulation of the levels of a neuropeptide, the clinical significance of its inhibitors as drugs and the goals of the work presented in this thesis.

1.1 Enzymes as Drug Targets

Enzymology is a discipline over 150 years old; however, since early studies concerned bacterial and yeast enzymes, the physiological importance of enzymes in relation to human diseases and disorders was not recognized until the turn of the century. In many pathological conditions, one or more physiological processes are altered in some manner. To regain the normal physiological state of the patient, one may attempt to alter discrete metabolic processes exogenously by the use of drugs acting on enzymes involved in the metabolic reactions of interest. Many drugs are compounds which act as inhibitors of specific

enzymes in metabolic pathways. Their administration allows control of the flux of metabolites through that particular metabolic pathway resulting in a regulation of a given physiological process(es).

Although a multitude of enzymes are involved in the normal functioning of mammalian cells, only a small proportion of these are likely to be of any use as potential drug targets. Some enzymes are fairly non-specific with respect to substrate, (e.g. mammalian alcohol dehydrogenase, [Winer, 1958]) so that inhibition or inactivation would not allow modulation of one specific process of metabolism, which is what is desired. Other enzymes have specific substrates but the end-product of the reaction, or series of reactions, is needed for several different physiological processes. For example hexokinase is a regulatory enzyme of glycolysis but inhibitors such as arsenicals are poisons rather than drugs because hexokinase is required for the metabolic usage of glucose as an energy source; a source upon which many other physiological processes depend. The problem in both cases is one of specificity. Enzymes suitable as drug targets must operate at key points in metabolism having specific substrates and end-products that interact with or affect only one (or few) process(es).

In the treatment of infections, some drugs operate on the principle of differential sensitivity. The bacterial or viral target enzyme is much more sensitive to the drug than the corresponding mammalian enzyme, with the result that only the pathogenic organism is affected (killed or rendered innocuous). One such class of drugs used for decades in the treatment of bacterial infections are the β-lactams (e.g. penicillin). These drugs function by interferring with bacterial

cell wall synthesis (Tipper and Strominger, 1965; Wise and Park, 1965). They inhibit the transpeptidases involved in cross-linking the peptidoglycan chains of the wall while mammalian transpeptidases are unaffected. Thus, the drug distinguishes between microbial and mammalian transpeptidases.

1.2 Mammalian Proteases

1.2.1 Classification

Proteases may be classified as exopeptidases (hydrolysing terminal amino acid residues from proteins) or endopeptidases (hydrolysing internal peptide bonds of proteins). According to Hartley (1960), they may be categorised broadly into four major groups depending upon the nature of the active site residues involved in catalysis: the serine, thiol, carboxyl- and metallo-peptidases. An initial starting point in the search for inhibitors (drugs) for an enzyme can be obtained by knowing to which class the enzyme belongs and by knowing compounds which are inhibitors of enzymes representative of this class.

1.2.2 Metallo-peptidases

Proteases of this class contain a catalytically essential active site metal ion. They may in addition contain regulatory or structural metal ions. Metallo-peptidases may be further subdivided into the endopeptidases, the carboxypeptidases and the aminopeptidases. The latter two are exopeptidases; carboxypeptidases cleaving C-terminal residues from substrates and aminopeptidases residues from the N-terminus.

Metallo-proteases are inhibited by metal-chelating agents such as EDTA and o-phenanthroline and can be identified by a stoichiometric relationship between metal content and enzyme activity upon reconstitution of the apoenzyme. Most known mammalian metallo-peptidases are zinc enzymes.

1.2.3 Angiotensin Converting Enzyme

One zinc protease of clinical importance upon which much work has been done is the angiotensin converting enzyme (ACE). ACE is a dipeptidyl carboxypeptidase, cleaving His-Leu from the C-terminus of its physiological substrate, the decapeptide hormone angiotensin I (Figure 1).

Angiotensin I is produced by the action of renin on the plasma prohormone angiotensinogen. The octapeptide product of ACE action on angiotensin I, called angiotensin II, is an extremely potent vasoconstrictor, while angiotensin I is biologically inactive. Angiotensin II is degraded by angiotensinase A to the less active heptapeptide angiotensin III which is itself degraded by non-specific proteases. ACE also hydrolyses bradykinin, a potent vasodilator, so that inhibition of the enzyme may help control hypertension by decreasing the degradation of this hormone as well as by decreasing the production of angiotensin II. Inhibitors of ACE could then be potential drugs for the treatment of hypertension in some patients.

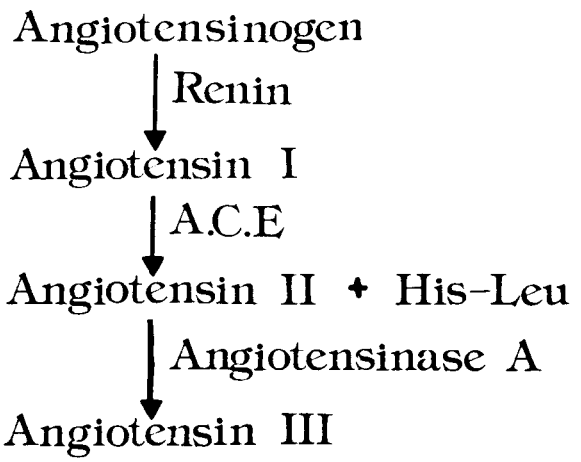
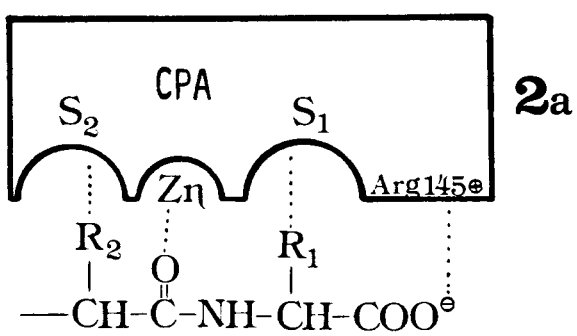


Figure 1- Angiotensin II biosynthetic pathway. (Reproduced in modified form from Ondetti and Cushman, 1982).



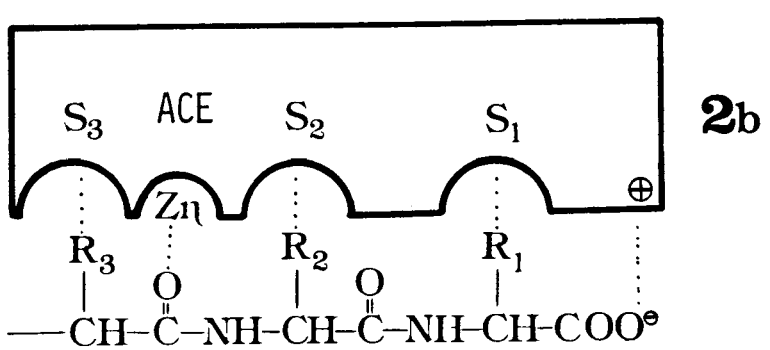


Figure 2- Active site models of CPA and ACE showing substrate binding. (Reproduced in modified form from Cushman and Ondetti, 1980).

1.2.4 Systematic Design of a Drug

ACE is a membrane bound enzyme found predominantly in the pulmonary vasculature although some is also present in plasma. The original purification procedures are tedious (Cheung and Cushman, 1973), although an affinity chromatography method is now available (Pantoliano et al., 1984). However, pancreatic carboxypeptidase A (CPA) is very similar to ACE in several structural and enzymatic properties, such as zinc content and amino acid residues present in the active site (Bünning et al., 1978; Soffer, 1981). CPA had then been studied for many years and was one of the best characterised zinc proteases known (Hartsuck and Lipscomb, 1971; Lipscomb, 1980; Vallee et al., 1983) as well as being commercially available in pure, crystalline form. This led researchers to use CPA as a prototype enzyme for the design of ACE inhibitors using a rational structure-function approach (Ondetti and Cushman, 1981; 1982).

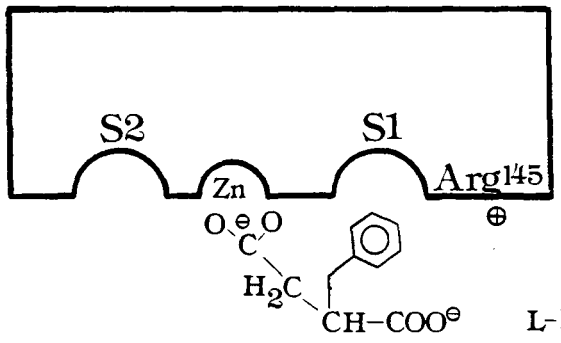
The active site of ACE was postulated to resemble that of CPA, except that a dipeptide is cleaved from substrates instead of a single amino acid residue (Figure 2). In the case of CPA, R_1 must be a hydrophobic group for tight binding of inhibitors or rapid hydrolysis of substrates, while L stereochemistry is a prerequisite. The nature of R_2 is not as crucial, but hydrophobic groups are best. The zinc metal polarises the scissile amide bond of substrates via the oxygen (Lipscomb, 1980), facilitating hydrolysis. Studies on ACE using peptide-inhibitors isolated from snake venom (Ferriera, 1965; Cushman et al., 1973) had shown that the best substituents were R_1 = pro, R_2 = ala and R_3 = aromatic or branched aliphatic. Unfortunately, these

potent inhibitors were rapidly degraded *in vivo* so that they were not useful as drugs. Biologically active inhibitors were desired and the finding of Byers and Wolfenden (1973) that L-benzylsuccinate is a strong, competitive inhibitor of CPA resulted in the design of analogous inhibitors for ACE.

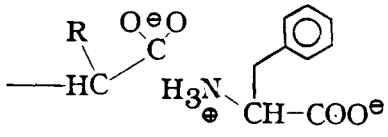
Byers and Wolfenden proposed that the tight binding of their inhibitor to CPA was due to its bi-product analogue nature. The functional groups of both of the products of the hydrolysis reaction are combined in one molecule to obtain maximal binding energy without the entropically unfavourable condition of binding two molecules independently and simultaneously (Figure 3). In addition, the C4 carboxyl group is a moderately good ligand for the active site zinc.

Succinyl-L-proline was the first such bi-product analogue inhibitor tried on ACE. Since it proved to be a moderately weak inhibitor, a series of derivatives of this compound was then synthesised (Cushman et al., 1977), each member differing in some structural aspect of the molecule, and were tested for inhibition. The largest increase (1,000-fold) in potency was observed upon replacement of the zinc-liganding carboxyl group with a mercapto moiety, a stronger zinc ligand. In this manner the drug captopril (D-3-mercapto-2-methyl-propanoyl-L-proline) was designed, found to have a K_i of 1.7 nM and found to be an orally effective antihypertensive agent.

Whereas the vast majority of drugs have been discovered more or less by serendipity or via folk remedies, captopril represents the systematic design of a drug by using knowledge of the molecular mechanism as well as substrate and inhibitor binding preferences of the



L-Benzylsuccinate



substrate hydrolysis products

Figure 3- Bi-product nature of L-Benzylsuccinate. (Reproduced in modified form from Cushman et al., 1977).

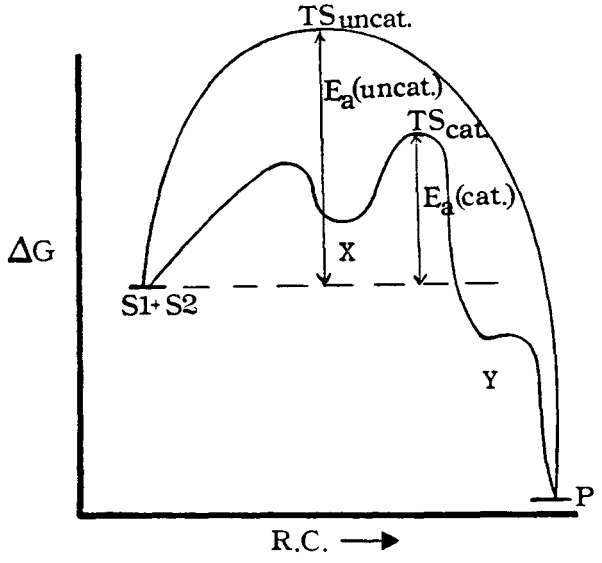


Figure 4- Energy profiles of uncatalysed and catalysed reactions. S1, S2 = substrates; P = products; X, Y = intermediates; TS = transition state; E_a = activation energy. (Reproduced in modified form from Fersht, 1977).

target enzyme. The extremely tight binding of captopril suggests that it is a transition state analogue inhibitor instead of a bi-product analogue inhibitor, though it is difficult to draw a fine line of distinction between these two types of inhibitors.

1.3 Transition State Theory

Transition state theory (Pelzer and Wigner, 1932) examines the thermodynamic pathway followed by an enzyme catalysed reaction. Along a reaction pathway there will exist a point at which the free energy of the system reaches a maximum value, called the transition state. "In the transition state chemical bonds are in the process of being made and broken" (Fersht, 1977). Since the rate of decomposition of the transition state is determined by the vibrational frequency (kT/h) of the bond being broken (Eyring,1935), the transition state is too fleeting to be detected. The nature of the transition state is inferred from knowledge of detectable intermediates preceding and succeeding it.

The reason why enzymes (or for that matter, any catalyst) cause such large increases in the reaction rate as compared to the uncatalysed reaction rate is that they provide a different thermodynamic pathway to go from reactant(s) to product(s) by stabilising the transition state. In the catalysed pathway, the activation energy (E_a), which is the energy required to overcome the rate-limiting step of the pathway, is lower than the activation energy of the uncatalysed reaction. Figure 4 illustrates energy profiles for uncatalysed and catalysed reactions. The transition state of the catalysed reaction is

lower in free energy than that of the uncatalysed reaction. Points X and Y represent meta-stable intermediates that exist along the pathway and since small energy barriers exist to their decomposition, they may possibly be detected or isolated. Bi-product analogue inhibitors resemble the intermediate Y while bi-substrate analogue inhibitors could resemble intermediate X. Since by the Hammond postulate (Hammond, 1955) these species should more closely resemble the transition state than either P or $S_1 + S_2$, they will bind to the enzyme with a higher affinity than the product(s) or substrate(s).

From known organic reaction mechanisms it is quite possible to speculate on the catalytic mechanism of many enzymes and to guess at the transition state. The design of stable compounds which resemble as closely as possible the supposed transition state yields extremely potent inhibitors since the enzyme affinity for the transition state is the largest of any of the species on the pathway. Transition state analogue inhibitors are potent, reversible, competitive inhibitors but another class of inhibitor, specific-irreversible inhibitors, can prove to be yet more potent.

1.4 Inhibition Modes

Enzyme inhibitors may be classified as reversible or irreversible. Reversible inhibitors bind to enzymes via non-covalent forces such as electrostatic and hydrophobic interactions. Dialysis of the solution restores full enzymatic activity as the inhibitor is diluted to a negligible concentration. Using the Lineweaver-Burk double reciprocal plot (Lineweaver and Burk, 1934) four basic types of reversible inhibition can be observed: competitive, noncompetitive, mixed (or partial noncompetitive) and uncompetitive (Cleland, 1970). Competitive inhibitors bind to the same site (or at least a portion thereof) as does substrate. Because of the specificity displayed by the active site towards substrate, the most useful drugs are competitive inhibitors in order to achieve the potency and specificity required.

Irreversible inhibitors form covalent bonds with the enzyme and cause a permanent inactivation of the enzyme activity which is not restored upon dialysis. The extent of inhibition is dependent on the rate of this bond formation. They can further be subdivided into non-specific and specific inhibitors. Non-specific irreversible inhibitors are usually substrate analogues (to obtain a partial degree of specificity) containing a reactive functional group. Hopefully, because of its resemblance to substrate, the compound will tend to be preferentially directed to the active site by normal reversible, non-covalent forces before the reactive group forms a covalent bond to the protein. The disadvantage of these types of inhibitors, especially when viewed

as potential drugs, is that the functional group is so reactive that it reacts indiscriminantly and a large degree of specificity is lost.

Specific irreversible inhibitors are the most promising class of drugs as far as specificity and potency are concerned (Abeles, 1978; Rando, 1978). Aspirin is the most well-known example of this class of inhibitor, acting on cyclooxygenase (Roth et al., 1975). These inhibitors contain dormant reactive functional groups that become activated by the catalytic mechanism of the enzyme. The enzyme attempts to use the compound as a substrate and along the catalytic pathway the reactive moiety is exposed which can then inactivate the enzyme. Such compounds are therefore known as suicide substrates (or suicide inhibitors or k_{cat} inactivators or mechanism-based inhibitors). The high degree of specificity comes in part from the substrate recognition factors and in larger part from the fact that the catalytic mechanisms of different enzymes are not the same. The efficacy of a suicide substrate is determined not only by its dissociation constant (K_i) but also by the rate constant for conversion to reactive product and subsequent covalent bond formation within the enzyme active site. Excellent reviews on the strategies involved in the design of suicide inhibitors are provided by Abeles and Maycock (1976) and Walsh (1982).

1.5 Yonetani-Theorell Kinetics

In order to understand more fully the mode in which inhibitors bind to an active site of an enzyme, it is useful to compare the simultaneous binding of two inhibitors which appear to bind in a similar fashion (i.e. competitively). The Yonetani-Theorell (Yonetani and Theorell, 1964) kinetic analysis may be used to this end. The degree of interaction between two inhibitors can be established by this multiple inhibition analysis. Graphically, it is similar to the Dixon plot (Dixon, 1953) where the inverse of the reaction velocity is plotted against inhibitor concentration at a fixed substrate concentration and a family of lines is obtained by assaying at several different substrate concentrations. In the Yonetani-Theorell plot, a family of lines is obtained by assaying at several different concentrations of a second inhibitor while keeping the substrate concentration constant for all. The intersection point of the lines occurs at an abscissa value of $-\alpha K_{i(I_1)}$ where $K_{i(I_1)}$ is the dissociation constant (under the assay conditions used) of the inhibitor designated I_1. The constant α is a measure of the interactions between I_1 and the second inhibitor, I_2. Knowing the value of $K_{i(I_1)}$, α can be calculated from the intersection point (Figure 5).

Four types of interactions may be discerned by this analysis:

1. $\alpha = \infty$ (a series of parallel lines, i.e. no intersection point) indicates mutually exclusive (competitive) binding between I_1 and I_2.

2. $\infty > \alpha > 1$ indicates repulsive or negative interactions

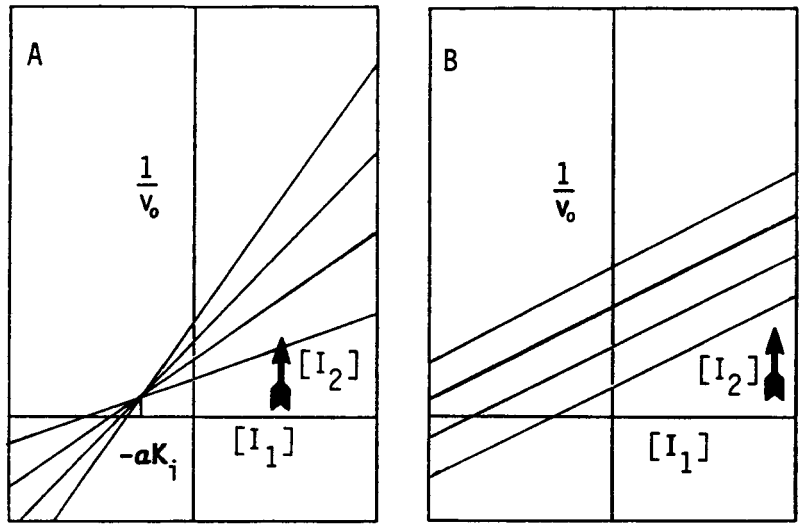


Figure 5- Yonetani-Theorell Kinetic Plot.
The inverse of the initial reaction velocity is plotted against the concentration of the first inhibitor (I_1) at constant substrate concentration. A family of lines is generated by increasing the concentration of a second inhibitor (I_2).
A. The intersection point shown is $-\alpha K_i$, where K_i is that of I_1 and $\infty > \alpha > 0$.
B. A family of parallel lines indicates $\alpha = \infty$.
(Reproduced in modified form from Yonetani and Theorell, 1964).

between binding of I_1 and I_2.

3. $\alpha = 1$ indicates completely independent binding of I_1 and I_2 (noncompetitive binding of I_1 with respect to I_2).

4. $1 > \alpha > 0$ indicates positive co-operativity (synergism) between binding of I_1 and I_2.

This analysis allows the investigation of interactions within subsites of the active site and determination of the mode of binding of different functional groups and different classes of inhibitors.

1.6 EDTA Inactivation Kinetics

Another manner in which the interactions between inhibitors and MAP may be studied is by the degree of protection provided by an inhibitor against EDTA inactivation of the enzyme (Chan et al., 1982; Chan, 1983). (For the derivation of kinetic equations for a postulated mechanism, see Appendix.)

EDTA chelates Zn^{2+}, which is required for enzymatic activity, thereby inactivating MAP. Incubation of MAP in 10 mM EDTA at 25°C causes no noticeable inactivation over a period of several hours. Incubation at 37°C however gives rise to > 90% inactivation within a few hours. It is proposed that spontaneous dissociation of Zn^{2+} from the enzyme increases (i.e. k_1 increases, see Appendix) with increasing temperature and EDTA then binds the freed Zn^{2+} in solution. In the presence of some inhibitors, the observed rate of inactivation is decreased; these inhibitors are said to protect MAP.

1.7 Microsomal Aminopeptidase

The goal of this work was to investigate the mode of binding to the active site of microsomal aminopeptidase[1] (E.C. 3.4.11.2) of various inhibitors and to determine structure-activity relationships. Microsomal aminopeptidase was chosen as a prototypical aminopeptidase to study because of its commercial availability and sensitive, facile assay (Tuppy et al., 1962). Hopefully, knowledge gleaned from this prototypical enzyme will be of use in the future design of pharmacologically important drugs in the area of analgesia; in the same manner as CPA was of use for the design of inhibitors of ACE which are therapeutic drugs for the treatment of hypertension.

1.7.1 Physiological Roles of Aminopeptidases

While the carboxypeptidase and endopeptidase (e.g. thermolysin, a bacterial zinc protease) metallo-proteases have been extensively studied, much less work has been done on aminopeptidase metallo-proteases, although their presence has been known for almost six decades (Linderstrøm-Lang, 1929; 1930). Little is known of the active sites of these enzymes and the mechanism of hydrolysis is unknown.

Aminopeptidases were used mainly in protein chemistry for sequencing N-terminal residues of proteins (Smith and Hill, 1960; Light, 1967). Recent findings have implicated aminopeptidases to be

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[1] Also known as leucine aminopeptidase (incorrectly), particulate aminopeptidase, aminopeptidase M, aminopeptidase N and neutral aminopeptidase.

involved in the degradation of enkephalins in brain tissue (Vogel and Altstein, 1980; Shimamura et al., 1983; Chaillet et al., 1983; Gros et al., 1985). Enkephalins are peptide neurotransmitters, of which the opiates are agonists, involved in the alleviation of pain (Hughes et al., 1975). The problems with using opiates as analgesic drugs are that patients acquire tolerance, requiring larger and larger amounts of drug to be administered for analgesia and that they are addictive. A non-addictive, potent analgesic would be a tremendously valuable drug to medicine. Inhibitors of the aminopeptidases that degrade endogenous enkephalins have the potential to be such drugs, allowing the body's natural 'pain-killers' to operate maximally.

1.7.2 General Properties

The enzyme was first isolated and purified by Pfleiderer and Celliers (1963), (see also, Pfleiderer, 1970), later improvements in purification procedures being introduced by Wachsmuth et al., (1966a). MAP has a relative molecular mass of approximately 280,000 (Auricchio and Bruni, 1964), containing 20% carbohydrate by mass (Wacker et al., 1971).

The quaternary structure of the protein is not resolved as yet, but is thought to be dimeric (Wacker et al., 1976). SDS denaturation of the M_r 280,000 membrane-bound protein yields a single M_r 140,000 subunit. The presence of two moles of catalytically essential zinc per mole enzyme (Lehky et al., 1973) suggests the existence of two subunits each containing one active site, though this is not proven. Previous reports on the subunit structure of MAP have proposed a decameric

arrangement consisting of two distinct subunits (Wachsmuth, 1967a). Microsomal aminopeptidase appears to be a transmembrane protein. The hydrophilic portion containing the active site may be released by trypsin digestion leaving an approximately M_r 4,000 anchor in the membrane (Louvard et al., 1975; 1976; Benajiba and Maroux, 1980; Ward, 1984).

1.7.3 Substrates and Physical Properties

MAP hydrolyses amino-terminal residues from peptide substrates, having a preference for residues with large aliphatic or aromatic side chains (Wachsmuth et al., 1966b; Pfleiderer et al., 1964). Only L amino acid residues are hydrolysed. Alanine derivatives are most rapidly hydrolysed (highest k_{cat}) while the larger, hydrophobic amino acids are more tightly bound (lower K_m). The result is that the second order rate constant, k_{cat}/K_m, varies only a few fold for most of the nonpolar amino acids (Wachsmuth et al., 1966a; Wachsmuth, 1966; Pfleiderer et al., 1964). This is in contrast to the cytosolic aminopeptidase (E.C. 3.4.11.1) which exhibits a 1,000-fold variation in k_{cat}/K_m. MAP is stable over a wide pH range, 3.5 to 11 (Wachsmuth et al., 1966a), the pH optimum for activity being 7.0-7.5 at which it is stable at temperatures up to 65°C. The enzyme is irreversibly denatured by long chain alcohols and is insensitive to the $MgCl_2/MnCl_2$ activation which is found for the cytosolic enzyme.

1.7.4 Active Site Residues

Histidine (Pfleiderer and Femfert, 1969) has been found to be essential to the catalytic activity and is likely present in the active site. Tyrosine (Wachsmuth, 1967b; Femfert and Pfleiderer, 1969; Femfert et al., 1972) was also found to be essential for activity. p-Mercuriphenylsulphonate and iodoacetate were non-inhibitory, indicating that no essential cysteine residues are likely present (Pfleiderer et al., 1964).

Enzyme inactivation increases sharply at pH values less than six and is likely due to acid facilitated dissociation of zinc ion from the active site. Since histidine residues are present in the active site, it is possible that a histidine(s) with a pK_a near six acts as a zinc ligand.

1.7.5 Metal Ion Role

Removal of metal from the holoenzyme (MAP) increases enzyme sensitivity to denaturing conditions (Lehky et al., 1973) implying a role for the zinc in structure stabilisation. Activity can be restored to the apoenzyme by the addition of Zn^{2+}, Co^{2+}, Cu^{2+} or Ni^{2+} and restoration of activity is directly proportional to the amount of metal added up to 2 moles/mole protein.

The Co^{2+}-, Cu^{2+}- and Ni^{2+}-metallo-enzymes are readily inactivated in the presence of EDTA at 25°C, indicating a dissociation of the metal from the protein. Zn^{2+}-MAP however does not readily inactivate under the same conditions, indicating a much higher affinity of the enzyme for zinc than the other metals. The Co^{2+} and Cu^{2+} species of

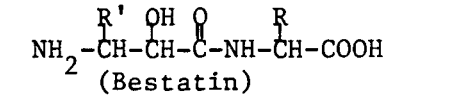
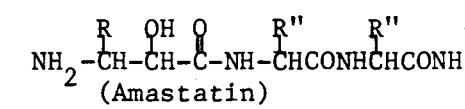
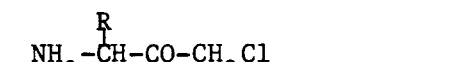
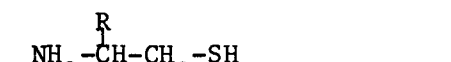
enzyme have a higher specific activity than the Zn^{2+} enzyme, while that of the Ni^{2+} enzyme is lower. Incubation of the metalloenzymes in the presence of excess zinc rapidly (less than five minutes) causes the specific activity to assume the value for that of the zinc enzyme, showing a fast exchange of Co^{2+}, Cu^{2+} and Ni^{2+} for Zn^{2+}.

1.7.6 Inhibitors

Among the first specific inhibitors of MAP was leucine chloromethyl ketone, Table I, which was synthesised in the hope that it would be an irreversible inhibitor as was the case for haloketones with the serine proteases. Surprisingly, it was found to be a good reversible inhibitor. The naturally occurring inhibitor bestatin (from a bacterial culture) was then discovered and a variety of analogues were later synthesised (Rich et al., 1984). The compound amastatin was found to be the most potent, with K_i = 20 nM.

A variety of inhibitors, all based on L-leucine, have been synthesised, using different zinc liganding moieties in place of the carboxyl group. L-leucine itself is an inhibitor (product inhibition) with a K_i = 1.2 mM. As with CPA and ACE above, replacement of the carboxyl group with a more potent zinc ligand improved binding. The hydroxamate group is likely a bidentate zinc ligand as it is for thermolysin (Holmes and Matthews, 1981), explaining the strong binding of L-leucine hydroxamate. Boronic acids have been tried as a ligand group for Aeromonas aminopeptidase (a bacterial enzyme) and phosphonic amino acids may be useful as well for MAP since they have proven to be good inhibitors for ACE and thermolysin. One of the best liganding

TABLE I - Inhibitors of Aminopeptidase

Compound	K_i (μM)	Reference
Naturally occurring:		
 (Bestatin)	4.1	(Umezawa, 1976)
 (Amastatin)	0.02	(Rich et al., 1984)
Synthetic:		
 (L-leucine chloromethyl ketone)	12	(Birch et al., 1972)
 (L-leucinal)	0.76	(Andersson et al., 1982)
 (L-leucine hydroxamic acid)	12	(Chan et al., 1982)
 (L-leucinthiol)	0.022	(Chan, 1983)
 (1-butane boronic acid)	9.6*	(Baker et al., 1983)

R = $-CH_2-CH(CH_3)_2$ R' = $-CH_2-C_6H_5$ R" = $-CH(CH_3)_2$

*K_i value for Aeromonas Aminopeptidase, a bacterial enzyme.

groups are aldehydes which have recently been shown to inhibit as the gem-diol form (Andersson et al., 1985) in the case of L-leucinal. This would be a transition state analogue as shown in Figure 6. The best synthetic inhibitor for MAP is the corresponding thiol derivative of L-leucinol (L-leucinthiol) where, as was found for CPA and ACE, the mercapto moiety is a very strong zinc ligand.

1.7.7 MAP Inhibitors as Drugs

Bestatin has been found to have antinociceptive properties (Chaillet et al., 1983) and to be an inhibitor of enkephalin degradation (Cohen et al., 1983). Hydroxamates have also been shown to inhibit enkephalin degrading aminopeptidases (Blumberg et al., 1981; Coletti-Previero et al., 1982). L-leucinal was discovered to potentiate the analgesia induced by leu-enkephalin administration in rats (Davis et al., 1983). Recently, an enzyme has been identified in rat brain membrane which has enkephalinase activity and is apparently identical to microsomal aminopeptidase (Gros et al., 1985). These preliminary findings suggest that MAP inhibitors may find clinical applications as analgesics.

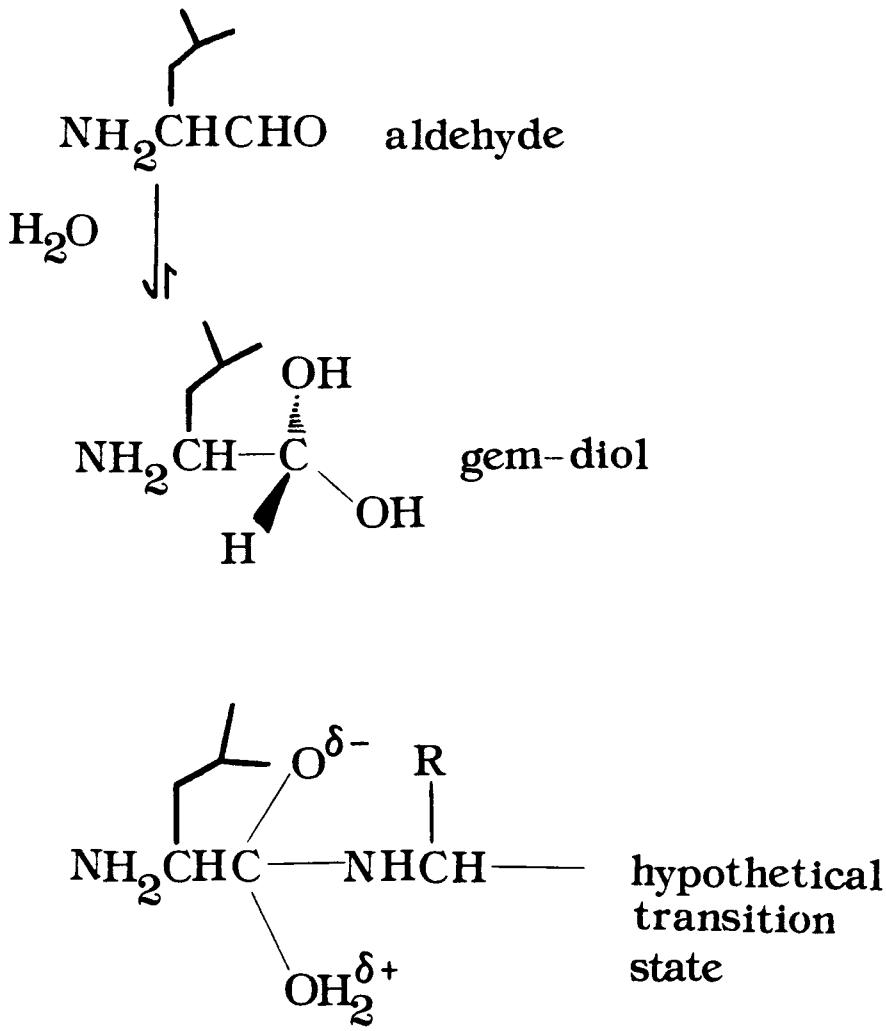


Figure 6- L-Leucinal; a transition state analogue inhibitor. The gem-diol form of the aldehyde resembles the postulated transition state.

1.7.8 Investigation of MAP Active Site

In order to realise the long-term objective of the development of potent, specific inhibitors for microsomal aminopeptidase, which may find clinical uses, it is first necessary to further characterise the active site of the enzyme. The kinetic methods described above (i.e. Lineweaver-Burk, Dixon, Yonetani-Theorell and EDTA inactivation) are used to this end. Derivatives of β-mercaptoethylamine are used to examine the structural requirements for optimal binding of mercaptoamines to the active site. Yonetani-Theorell kinetic analyses are used to locate subsites within the active site (i.e. specific regions of the active site responsible for binding the different, individual functional moieties of an inhibitor molecule). Interactions of a positively or negatively co-operative nature that occur between subsites is also explored using Yonetani-Theorell kinetics. The presence of multiple binding modes for an inhibitor as well as different binding modes occurring for different classes of inhibitors is investigated by Yonetani-Theorell and EDTA inactivation kinetics. Lastly, the prospects for inhibitor and drug design are discussed with respect to increasing their potency and specificity towards microsomal aminopeptidase.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Enzymes and Substrates

Porcine kidney microsomal aminopeptidase (MAP) (E.C. 3.4.11.2), (Type IV-S) a suspension in 3.5 M $(NH_4)_2SO_4$, pH 7.7, 10 mM $MgCl_2$, was obtained from Sigma Chemical Co., as was its substrate, L-leucine p-nitroanilide hydrochloride (LeupNA). Bovine pancreatic carboxypeptidase A (CPA) (E.C. 3.4.17.1) was also obtained from Sigma as an aqueous suspension (Type I) under toluene. Solid thermolysin (THL) (E.C. 3.4.24.4) and the CPA substrate 3-(2-furylacryloyl)-L-Phe-L-Phe-OH monohydrate (FAPP) came from Calbiochem-Boehring. The THL substrate, 3-(2-furylacryloyl)-Gly-L-Leu-NH_2 (FAGLA), was purchased from Peninsula Laboratories. The enzymes and substrates were stored at 5°C for more than a year without a noticeable loss of activity.

2.1.2 Enzyme Dilutions

Prior to each kinetic experiment, MAP and CPA were freshly diluted, approximately 10^2-fold and 10^5-fold respectively, into 100 mM Tris-HCl, pH 7.5, containing 10 µM $ZnCl_2$ and 0.2 mg bovine serum albumin per mL. Aliquots of these solutions were added to assay cuvettes to initiate the reaction, giving final concentrations of 1 µg/mL for MAP and 0.02 µg/mL for CPA in a total volume of 1 mL.

THL was weighed out and dissolved in 100 mM Tris-HCl, pH 7.2, containing 10 mM $CaCl_2$ to give a protein concentration of about 1 mg per mL. This solution could be kept at 5°C and used for several days before being discarded. Aliquots of this solution were used to initiate the enzyme reaction to give a final protein concentration of 3 µg/mL in a final volume of 1 mL.

2.1.3 Substrate Preparation

A stock solution of 10-20 mM LeupNA was prepared by dissolving it in water. Autohydrolysis increases at acidic or (particularly) alkaline pH values. This solution may be kept frozen at -20°C for up to two weeks, being thawed when needed and then refrozen.

A stock solution of about 1 mM FAPP was prepared by dissolving substrate in DMF and diluting 100-fold with 10 mM Tris-HCl, pH 8.5. Since the compound was supplied as the monohydrate, the FAPP concentration was more accurately determined by measuring the absorbance of a 1/100 dilution of the stock solution into methanol, (λ_{max} = 302 nm, $\varepsilon_{302,methanol}$ = 24,255). A 10 mM FAGLA stock solution was made by dissolving it in DMF and diluting 100-fold with water. The FAPP and FAGLA stock solutions were kept at 5°C for up to two weeks then discarded. All solutions were prepared using distilled, double-deionised water.

2.1.4 Inhibitors

L- and D-leucinthiol ({S}- and {R}-2-amino-4-methyl-1-pentanethiol), γ-mercaptopropylamine and 1,3-diamino-2-mercaptopropane were synthesized by Dr. M.V. Krishna (Pickering et al., 1985).

L-leucinamide hydrochloride, L-leucine hydroxamic acid, (D,L)-norleucine hydroxamic acid, glycine hydroxamic acid, acetohydroxamic acid, α-aminoisobutyric hydroxamic acid, (D,L)-2,3-diaminopropionic acid, (D,L)-α-alanine hydroxamic acid, β-mercaptoethylamine hydrochloride (cysteamine hydrochloride), isoamylamine, methylamine, ethylamine and selenocystamine dihydrochloride were obtained from Sigma.

D-leucine hydroxamic acid was from Enzyme Systems Products, (Livermore, CA). Ethylenediamine was a product of Fischer Chemical Co. while 1,3-diaminopropane was a reagent of MCB Chemicals. 2-Dimethylaminoethanethiol hydrochloride and 2-n-butylaminoethanethiol were purchased from Aldrich Chemical Co. β-Alanine was supplied by Eastman Organic Chemical Co.

2.1.5 Preparation of Hydrochlorides

Isoamylamine, ethylamine, methylamine, ethylenediamine and 1,3-diaminopropane were converted to the corresponding hydrochloride salts by dissolution in 6 M HCl and evaporation. The solids were then recrystallised from hot isopropanol (or isopropanol-water) upon standing at $5^{o}C$ overnight and were filtered, dried and stored in a dessicator.

2.1.6 Inhibitor Preparation

Amino acid hydroxamate inhibitor solutions were made by dissolving the solid in a small (less than 50 μL) volume of 2 M HCl and bringing to the final volume with water.

Commercially available mercaptoamines may contain disulphide due to autooxidation. The concentration of free thiol in inhibitor solutions made from these compounds may be determined using the DTNB assay (see Section 2.2.5). Amine concentrations may be determined by the TNBS assay as a double-check (see Section 2.2.6). Since 1,3-diamino-2-mercaptopropane dihydrochloride disulphide was a pasty, hygroscopic solid, a solution was prepared and the concentration determined by the TNBS assay. This was then reduced to the free thiol as described below (see Section 2.2.3).

For the determination of L-leucinthiol inhibition constants with MAP, reduction of the disulphide was achieved by a fifteen minute incubation of a 0.5 μM solution of the disulphide in 1 mM DTT. This inhibitor solution was added to the assay solutions containing 1 mM DTT, final concentration. For D-leucinthiol inhibition constants with MAP, reduction of the disulphide was achieved by addition of the disulphide solution to assay solutions containing 5 mM DTT and allowing a 5-10 minute incubation time before addition of enzyme.

2.1.7 Other Chemicals

Tris base and Tris hydrochloride, (D,L)-dithiothreitol, diphenylthiocarbazone (dithizone), picrysulphonic acid (TNBS), (2,2'-dithiobis-[ethylamine]) dihydrochloride (cystamine dihydrochloride), 2,4,6-trimethylpyridine (collidine), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and caproic acid ethyl ester were obtained from Sigma. β-Mercaptoethanol, sodium hydrosulphide, N-hydroxysuccinimide and dicyclohexylcarbodiimide were products of Aldrich. Analytical grade zinc dust came from Mallinckrodt, Inc. Ninhydrin was supplied by Pierce Chemical Co. Remaining chemicals and solvents were of reagent grade available from commercial sources.

2.1.8 Preparation of Synthetic Reagents

Caproic acid ethyl ester was hydrolysed to sodium caproate in a saturated solution of sodium hydroxide at room temperature, overnight with stirring. The hydrolysate was cooled in ice, then carefully and slowly acidified with small aliquots of concentrated sulphuric acid to pH less than three[2]. The acid solution was then extracted several times with diethyl ether, the ether fractions combined and evaporated under reduced pressure. n-Caproic acid was obtained as a clear, colourless, viscous liquid.

Dicyclohexylcarbodiimide (DCCD) was purified by dissolution into warm $CHCL_3$ which was stoppered and left to cool to $5^{o}C$. The

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[2] N.B. This reaction was extremely vigorous and splashing occurs unless very small aliquots of acid are added slowly.

precipitated dicyclohexylurea (DCU) was then filtered from the solution and the solvent evaporated to give a pasty solid DCCD which was essentially free of DCU (private communication from Dr. G. Gerber).

N-hydroxysuccinimide was dried by dissolution in methanol-benzene and evaporation of the benzene-water azeotrope under reduced pressure.

2.1.9 Spray Reagents

The hydroxamate spray reagent consists of 5% $FeCl_3$ in 0.5 M HCl. Hydroxamates appear as red-brown or purple spots upon heating the TLC plate (Lipmann and Tuttle, 1945).

The DTNB spray reagent (Dennis et al., 1986) is made by dissolving 0.5 g DTNB in 25 mL 90% methanol:10% water. After spraying, exposure to pyridine vapour causes reduced mercaptans to appear as yellow spots. Thioesters appear as yellow spots after exposure to ammonia vapour.

The ninhydrin spray reagent is prepared by dissolving 1.0 g ninhydrin in 500 mL n-butanol. To 25 mL of this is added 25 µL (0.1%) collidine (Woiwood, 1949). This solution is stable for several days at 5°C. After spraying and heating of the plates, primary amines appear as purple spots and mercaptoamines as yellow spots.

2.2 Methods

2.2.1 Enzyme Assays

Standardised assay conditions of 100 mM Tris-HCl pH 7.5, 0.3 M NaCl and 25°C were employed for all three enzymes, allowing direct comparison of inhibition constants since buffer effects, pH, ionic strength and chloride ion concentration in particular would not vary significantly from one assay to another. All solutions were prepared using distilled, double-deionised water. MAP activity was monitored spectrophotometrically by recording the increased absorbance at 405 nm as LeupNA was hydrolysed (Tuppy et al., 1962). CPA was assayed by recording the decrease in absorbance at 335 nm upon hydrolysis of FAPP (Peterson et al., 1982). A Cary 118 double beam recording spectrophotometer was used for assaying MAP and CPA, with a chart speed of 100 to 200 sec./inch and an absorbance range of 1.0 to 0.2 AUF. Typically, five samples were run simultaneously with each cell individually zero-suppressed in order to separate the chart tracings. Sample reading times of one to five seconds were used for a total elapsed reaction time of five to ten minutes. THL activity was followed by recording the hydrolysis of FAGLA at 345 nm (Feder, 1968) on a Gilford 2400 single beam recording spectrophotometer for the initial two minutes of reaction. (The reaction becomes non-linear after this time). All THL assays were performed by Dan Miller.

2.2.2 Kinetic Experiments

Initial reaction velocities were analysed by Lineweaver-Burk (Lineweaver and Burk, 1934) and Dixon (Dixon, 1953) plots to evaluate K_is and mode of binding of the inhibitors. Linear regression was applied to all data to yield best-fit lines. Assays were done in duplicate or triplicate at four or five substrate concentrations and K_is redetermined on separate days, the average value then being taken and the normal standard deviation about the mean determined using the formula:

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(\bar{x} - x_i)^2}{n - 1}}, \quad \text{where } 3 \leq n \leq 5$$

To determine mercaptoamine inhibition constants it was necessary to include an antioxidant in the assay mixtures. For MAP and CPA assays, 0.5 mM DTT was used while 2 mM β-mercaptoethanol was used for THL assays.

Substrate concentration ranges used were: 0.4 to 2.0 mM for MAP, 0.05 to 0.2 mM for CPA and 0.4 to 1.0 mM for THL. Under the assay conditions used, the K_m values of the substrates fell within these ranges. Four or five inhibitor concentrations were used when experimentally possible.

Yonetani-Theorell analyses (Yonetani and Theorell, 1964) for MAP inhibitors were performed in the same manner as Dixon analyses except that the substrate concentration was held constant at 1 mM and the concentration of the first inhibitor varied over four or five concentrations of a second inhibitor. The data was analysed by linear regression. The intersection point of each pair of lines was calcul-

ated from the mathematical equations generated by the linear regression analysis. The best-fit intersection point was taken as the average value of all the determined intersection points. Where repeated determinations of α were performed, the average value is given along with the normal standard deviation about the mean, calculated as above. The upper detection limit for the determination of α is approximately ten. Therefore, where the value of ∞ is assigned to α, it is meant that $\alpha > 10$. In a practical sense, as the value of α for a pair of inhibitors becomes larger than ten, the two compounds are for all intensive purposes completely, mutually competitive. This is because α is really an index of the degree of competition between simultaneous binding of two inhibitors and as α becomes larger than ten the competitive nature of the interaction approaches one of mutually exclusive binding.

EDTA inactivation experiments (Chan et al., 1982; Chan, 1983) were initiated by addition of diluted enzyme to the inactivation solution of 100 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 6 mM EDTA at several different concentrations of inhibitor (plus DTT if required), to give 20 µg MAP/mL, (all final concentrations). Incubation was carried out in a 37°C water bath and 50 µL aliquots of inactivation solution removed at approximately fifteen to twenty minute intervals for two to three hours. These aliquots were assayed immediately at 25°C by diluting 20-fold into the standard assay solution containing 1 mM LeupNA. This dilution into 25°C assay solution stops the EDTA inactivation process as well as diluting the inhibitor concentration 20-fold.

Prior to use, amine hydrochloride inhibitor solutions were

adjusted to pH 7.5 with NaOH and were then assayed in 0.1 M Tris-HCl buffer, pH 7.5; 1 M NaCl solution being added to the assay cuvette to bring the total chloride concentration to 0.3 M. In the cases where the inhibition constants are relatively small (less than 5 mM), this addition of NaCl need not be done since the addition to the assay of inhibitor at concentrations of four to five times K_i does not appreciably increase the total chloride concentration.

2.2.3 Disulphide Reduction

The disulphide hydrochloride derivative of a mercaptoamine inhibitor (amounts as small as 1-2 mg can be used) was weighed out into a 5 mL beaker and dissolved in 0.5 mL 2 M HCl. Zinc dust was added to obtain a vigorous H_2 evolution while the solution was rapidly stirred. After fifteen minutes, reduction was complete (as judged by by silica gel TLC using DTNB and ninhydrin sprays) and the acid solution remained stable for at least four hours.

Immediately before use, this solution was titrated to pH 2-3 using a Radiometer PHM63 Digital pH-meter equipped with microelectrodes by the addition of 10 µL aliquots of approximately 4 M aqueous NaSH while stirring rapidly. The white precipitate ZnS was removed by filtration on a Millipore 0.45 µM filter attached to a 5 cc. syringe. Removal of Zn^{2+} was necessary since at millimolar concentrations it will inhibit MAP. The solution was now brought to a convenient final volume and the mercaptoamine concentration calculated assuming no loss of disulphide. The solution was now degassed of H_2S by brief application of vacuum.

The pH of this inhibitor solution should be between two and four, otherwise it will either be too acidic to add to the assay cuvettes or reoxidation to the disulphide may become a problem. If necessary, a very small amount of concentrated HCl can be added to acidify the solution while negligibly changing the total volume. The concentration of NaCl produced in the solution by this procedure was in the range of 200 mM so that upon dilution into the assay cuvettes, the final chloride concentration will not change appreciably (5-10%).

Cystamine dihydrochloride was reduced by this procedure to cysteamine and the K_i obtained was within 20% of the value previously determined for pure cysteamine. No detectable level of Zn^{2+} (i.e. < 1 μM) was found in the reduction solution using the dithizone assay for zinc.

Selenocystamine dihydrochloride was reduced *in situ* with DTT, (> 200-fold excess).

2.2.4 Synthesis of n-Caproic Hydroxamic Acid

The hydroxamic acid synthesis of Nishino and Powers (1978) was used. n-Caproic acid, 4.0 g (34.4 mmol), was weighed out into a 250 mL round-bottomed flask using a pasteur pipette. N-hydroxysuccinimide, 5.3 g (43 mmol), was added and the mixture dissolved in 50 mL dry dioxane. DCCD, 8.9 g (43 mmol), was weighed out into a flask with a pasteur pipette by first melting the solid in the bottle in a 60°C water bath. Dry dioxane (10-20 mL) was added to this and the mixture transferred to the n-caproic acid solution. A drying tube was attached and the reaction allowed to proceed at room temperature overnight with stirring.

Hydroxylamine hydrochloride, 11.1 g (160 mmol), was dissolved in 40 mL dry DMF in a 250 mL round-bottomed flask and 23 mL (165 mmol) triethylamine (distilled) added. The dioxane solution was filtered to remove precipitated DCU and the filtrate added to the DMF solution. A drying tube was attached and the reaction left at room temperature 24 hours with stirring.

Evaporation under reduced pressure was performed to remove solvent. The residual oil was acid washed with 3 M HCl and extracted several times with diethyl ether. The ether fractions were combined and dried over solid $MgSO_4$, then evaporated to give a viscous red-orange oil. The majority of unreacted n-caproic acid (bp. 202°C) was removed by overnight evaporation at 90°C using an oil pump vacuum apparatus. Silica gel TLC (10:90:3 = methanol:diethyl ether:formic acid, eluent) showed one major spot at R_f = 0.70 in iodine vapour which was positive for the hydroxamate spray test. Minor spots appeared at the origin in iodine vapour which were removed after silica gel column purification using the above solvent system.

Upon standing at -20°C for several weeks, the oil solidified and remained solid at room temperature. The product was twice recrystallised from ethyl acetate-hexanes to give a white solid, pure as judged by TLC, mp. 64-65°C, in 10% yield. The ^{1}H-NMR spectrum ($CDCl_3$) showed: δ 0.9 (t,3H,methyl,J = 7 Hz), δ 1.0-1.8 (m,6H,methylene), δ 2.1 (t,2H,methylene), δ 8.2 (s,broad,2H,NHOH,D_2O exchangeable). The IR spectrum ($CHCl_3$) confirms the identity of the product as n-caproic hydroxamic acid; 3420 cm^{-1} (OH stretch), 3150-3350 cm^{-1} (broad, NH) and 1655 cm^{-1} (carbonyl).

2.2.5 DTNB Assay for Mercaptans

A standard solution may be prepared from mercaptosuccinic acid, a stable, non-hygroscopic solid. Up to 100 µL of the thiol solution (which should be between 0.1 and 50 mM) is added to 900 µL of 0.1 M Tris-HCl, pH 8.0; 1 mM EDTA; 1.1 mM DTNB. (DTNB may be initially dissolved in 90% methanol:10% H_2O and should be freshly diluted into a 10X concentrated Tris-EDTA solution which is then diluted with water to the final concentrations indicated.) The final volume is made to 1 mL with water. After 10 minutes, the absorbance reading at 412 nm is taken (Dennis et al., 1986).

2.2.6 TNBS Assay for Amines

A standard curve should be prepared using a compound similar to the unknown to be determined. Up to 200 µL of the amine solution (containing 0.05-0.10 µmol $R-NH_2$) is mixed with 200 µL 0.2 M sodium borate, pH 8.75 and 100 µL 0.2% TNBS (freshly made). The total volume is brought to 500 µL with water. This solution is incubated at 50°C for two hours, then 500 µL 0.5 M sodium acetate, pH 5.0; 10% SDS is added, the solution vortexed 30 seconds and the absorbance reading at 335 nm taken (Habeeb, 1966; as modified by Chan and Enns, 1981).

2.2.7 Zn^{2+} Determination (Dithizone Method)

This procedure was used to ensure zinc removal after the ZnS precipitation step of the disulphide reduction (see above). For the purposes required here, a semi-quantitative assay was used (modified from Barnes, 1951).

Standard solutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M $ZnCl_2$ are prepared from a stock solution of 100 mM. One hundred µL of each of the standard solutions (plus a blank) or of the unknown solution is added to 5 mL 25% ammonium citrate, pH 8.5 in a large test-tube. To this is added a drop of phenolphthalein and concentrated NH_4OH to give a pink colour. Ten mL of 0.01% dithizone in CCl_4 and 10 mL 0.2% aqueous diethyldithiocarbamate (filtered) are added. The solution is vortexed for three minutes, the upper aqueous layer pipetted off and discarded. Ten mL of 0.01 M NH_4OH is added and the solution vortexed one minute. The aqueous layer is discarded and the previous step repeated. This step decomposes excess dithizone. The concentration of zinc in the unknowns may be compared to that of the standards by measuring their absorption at λ = 540 nm using a spectrophotometer. If the concentration is less than 10^{-4} M, this is satisfactory.

3. RESULTS

3.1 K_i Studies

3.1.1 Effect of pK_a of Amino Group on Binding

A rough correlation seemed to exist between decreasing pK_a of the amine group and decreasing K_i of the inhibitors in Table II. Increasing the length of the alkyl chain, as with isoamylamine, counteracted the increase in K_i caused by the larger pK_a of the amine group. By comparing methylamine to ammonia and to isoamylamine, it is seen that the amine group and hydrophobic side chain make approximately equal contributions to the total binding energy of these inhibitors.

The presence of an α-amino group, in addition to the β-amino group, resulted in a large decrease in the K_i, as seen by comparing β-alanine to (D,L)-2,3-diaminopropionic acid.

3.1.2 Zinc Ligand Strength

In Table III the relative potency of various zinc-liganding groups is shown. The order of increasing strength of the ligands is the same as that observed for other zinc proteases such as CPA, ACE and THL (Holmquist and Vallee, 1979; Galardy and Kortylewicz, 1984; Kam et al., 1979; Nishino and Powers, 1979; Cushman and Ondetti, 1980; Ondetti and Cushman, 1981):

$$R\text{-}H < R\text{-}OH < R\text{-}CONH_2 \simeq R\text{-}COOH << R\text{-}CONHOH << R\text{-}SH \leq R\text{-}SeH$$

TABLE II

Amine Inhibitors

Inhibitor	K_i (mM)±S.D.	Inhibition mode	pK_a (NH_2)[a]
NH_2-CH_3	500	nd[b]	10.62
$NH_2-CH_2-CH_3$	250 (± 2%)	c	10.64
$NH_2-CH_2-CH_2-CH_2-NH_2$	103	mn	10.65
NH_3	58 (± 10%)	c	9.25
$NH_2-CH_2-CH_2-NH_2$	19 (± 6%)	mn	7.52, 9.98
$NH_2-CH_2-CH_2-COOH$	47	mn	10.19
$NH_2-CH_2-\underset{\underset{NH_2}{|}}{CH}-COOH$	0.69	c	6.73 (α), 9.56 (β)
$NH_2-CH_2-CH_2-CH(CH_3)_2$	48 (± 6%)	mn	10.60

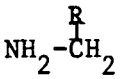
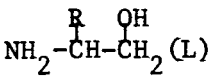
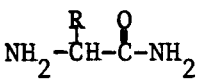
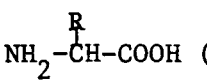
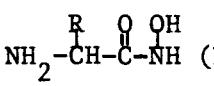
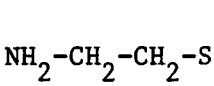
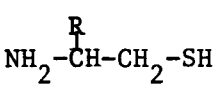
S.D. is standard deviation.
For assay conditions refer to METHODS.

[a] Literature values at 25°C (Sober, 1968).

[b] c = competitive, mn = mixed-noncompetitive, nd = not determined.

TABLE III

Binding Strength of Different Zinc Liganding Groups

Inhibitor	K_i(M)±S.D.	Inhibition mode
	4.8×10^{-2} (± 6%)	mn[a]
 (L)	1.8×10^{-2}	c
 (L)	7.9×10^{-3} (± 1%)	c
 (L)	2.9×10^{-3} (± 6%)	c
 (L)	2.6×10^{-5} (± 2%)	c
	8.4×10^{-7} (± 3%)	c
 (L)	5.1×10^{-8} (± 21%)	c

R = $-CH_2-CH(CH_3)_2$

S.D. is standard deviation.
For assay conditions refer to METHODS.
[a] c = competitive, mn = mixed-noncompetitive.

The potency of β-selenoethylamine (K_i = 0.84 μM) indicates that the selenol group is equal to or better than the thiol group as a zinc ligand, since the equivalent thiol compound (β-MEA) has a K_i of 3.2 μM (Table V). The pK_a of the selenol group in this compound is 4.90 (Martell and Smith, 1982) as compared to the pK_a of 8.6 (Sober, 1968) for the thiol of β-mercaptoethylamine. The amine groups of both compounds have pK_as of 10.8.

3.1.3 A Comparison of Hydroxamate Inhibitors

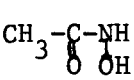
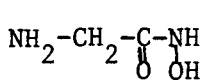
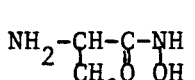
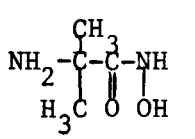
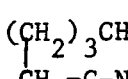
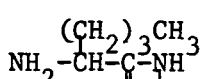
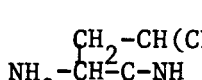
Decreasing the pK_a of the hydroxamate group two units, by the presence of an α-amino group (glycine hydroxamate vs. acetohydroxamate), decreased the binding strength (Table IV).

Increasing the size of the alkyl substituent of the α-carbon was seen to result in better binding. The larger K_i of α-aminoisobutyric hydroxamate as compared to (D,L)-α-alanine hydroxamate suggests that the second methyl group on the α-carbon is detrimental to binding.

The similarity in K_i values of (D,L)-norleucine hydroxamate, and L-leucine hydroxamate imply that this hydrophobic pocket does not show much preference towards one isomer over the other. The small differences between L- and D-leucine hydroxamate and n-caproic hydroxamate K_is illustrates that the amino group is not crucial to the binding of hydroxamate inhibitors and may not contribute much to the total binding energy.

TABLE IV

Hydroxamic acid Inhibitors

Inhibitor	K_i(M)±S.D.	Inhibition mode	pK_a(NHOH)[a]
	9.1×10^{-4} (± 5%)	mn[b]	9.40
	3.0×10^{-3} (± 19%)	c	7.40
 (D,L)	4.6×10^{-4}	c	-
	4×10^{-3}	nd	-
	1.2×10^{-4} (± 8%)	c	9.75
 (D,L)	3.2×10^{-5}	c	-
 (L)	2.6×10^{-5} (± 2%)	c	-
(D)	5.5×10^{-5} (± 3%)	c	-

S.D. is standard deviation.
For assay conditions refer to METHODS.

[a] Literature values, (Sober, 1968), at 25°C.

[b] c = competitive, mn = mixed-noncompetitive, nd = not determined.

3.1.4 Inhibition by β-Mercaptoethylamine Derivatives

The strong affinity of MAP for mercaptoamine inhibitors is exemplified by the tight binding of β-MEA and L-leucinthiol (Chan, 1983), (Table V). Because of the potency of β-MEA, despite its small size and simple structure, it was desired to explore the binding of mercaptoamines in more detail.

Mono- and di-alkylation of the amine group resulted in large (1,000-fold) decreases in the binding strength. Thus, in contrast to the hydroxamate series of inhibitors, the amine group of mercaptoamine inhibitors plays an important role in its binding. Alkylation of the amine group increased the pK_a, which is unfavourable, and introduced the possibility of steric hindrance effects.

Increasing the size of the molecule by one methylene unit to give γ-mercaptopropylamine was similarly unfavourable. This would result in an increased pK_a for the amino group and could also make the molecule too large for optimal binding of the thiol and amino groups.

The addition of an aminomethyl substituent on the β-carbon (2-mercapto-1,3-diaminopropane) increased the K_i by a factor of 53, as compared to β-MEA. It was initially hoped that the inclusion of an extra aminomethyl group in the structure might allow for additional binding to a second amine-binding subsite thought to be present in the active site. However, a second amino group proved to be unfavourable, at least when in the γ position.

Substitution on the α-carbon by a large, hydrophobic group (L-leucinthiol) gave an increase in binding of 63-fold. The effect of the stereochemistry of the substitution at the α-carbon was investi-

TABLE V

Specificity of Mercaptoamine Binding to MAP

Inhibitor	K_i (mM)±S.D. MAP mode	CPA mode	THL[c] mode
$NH_2-CH_2-CH_2-SH$	0.0032 c[a] (± 2%)	5.7 n (± 14%)	0.71 c (± 2%)
$CH_3(CH_2)_3NHCH_2CH_2SH$	2.0 c (± 7%)	2.8 n (± 13%)	0.18 n (± 2%)
$(CH_3)_2-NHCH_2CH_2SH$	> 5.0[b] nd	2.5 nd (± 15%)	> 5.0 nd
$NH_2-CH_2-CH_2-CH_2-SH$	> 5.0 nd	0.50 c	> 5.0 nd
$NH_2-CH_2-\underset{\underset{SH}{|}}{CH}-CH_2-NH_2$	0.17 c (± 19%)	-	-
$NH_2-\underset{\underset{CH_2-CH(CH_3)_2}{|}}{CH}-CH_2-SH$ (L)	0.000051 c (± 21%)	0.11 n (± 23%)	0.056 c (± 4%)
(D)	0.036 c (± 8%)	0.33 n (± 4%)	0.006 n (± 5%)

S.D. is standard deviation.
For assay conditions refer to METHODS.

[a] c = competitive, n = noncompetitive, nd = not determined.

[b] The upper limit for K_i determinations is 5 mM. At higher concentrations, the inhibitor could not be kept in the reduced state.

[c] Values determined by Dan Miller.

gated by comparing the D and L isomers of leucinthiol. L stereochemistry is preferred by at least a factor of 1,000 and perhaps even greater since the inhibition observed may actually be due, in part or in whole, to a suspected small (<0.14%) contamination of the D isomer by the L.

3.1.5 Specificity of Mercaptoamines towards MAP

The specificity of mercaptoamine inhibitors towards MAP was examined by comparison against CPA and THL, two other zinc-proteases. Whereas all the compounds of Table V were competitive inhibitors of MAP, they were mainly noncompetitive against CPA and THL. Binding was worst with CPA while THL bound the compounds with a strength intermediate between MAP and CPA. L-leucinthiol was 1,000-fold more potent as an inhibitor of MAP than of CPA or THL, while D-leucinthiol was a slightly better (7-fold) inhibitor of THL than MAP.

3.2 Yonetani-Theorell Kinetic Studies

3.2.1 Binding of the Amino Group

Ammonia was used as the marker for the amine-binding subsite(s), in the form of ammonium chloride. It is immediately evident that the binding of the amino moiety of the mercaptoamines is different from that of the hydroxamic acids (Table VI). The former exhibited mutually exclusive binding against ammonia while the latter could simultaneously bind with ammonia, indicating that the amino group of amino acid hydroxamates and mercaptoamines bind at different subsites.

TABLE VI

Binding to the Amine-Recognition Site

First Inhibitor		Second Inhibitor	α (±S.D.)	Interpretation[a]
$NH_2-\underset{R}{CH}-CH_2-SH$	(L)		∞[b]	X
	(D)		∞	X
$NH_2-CH_2-CH_2-SH$			∞	X
$NH_2-CH_2-CH_3$			∞	X
$NH_2-\underset{R}{CH_2}$		NH_3	∞	X
$NH_2-\underset{R}{CH}-\underset{O}{\overset{\|}{C}}-\underset{OH}{\overset{\|}{NH}}$	(L)		0.19 (± 21%)	+
	(D)		2.8 (± 4%)	-
$\underset{R'}{CH_2}-\underset{O}{\overset{\|}{C}}-\underset{OH}{\overset{\|}{NH}}$			0.16	+
$NH_2-CH_2-\underset{O}{\overset{\|}{C}}-\underset{OH}{\overset{\|}{NH}}$			0.17	+

$R' = -(CH_2)_3CH_3$ $\quad R = -CH_2-CH(CH_3)_2$

S.D. is standard deviation.
For assay conditions refer to METHODS.

[a] X = mutually exclusive, - = interference, + = synergism.

[b] The upper detection limit for α is approximately ten.

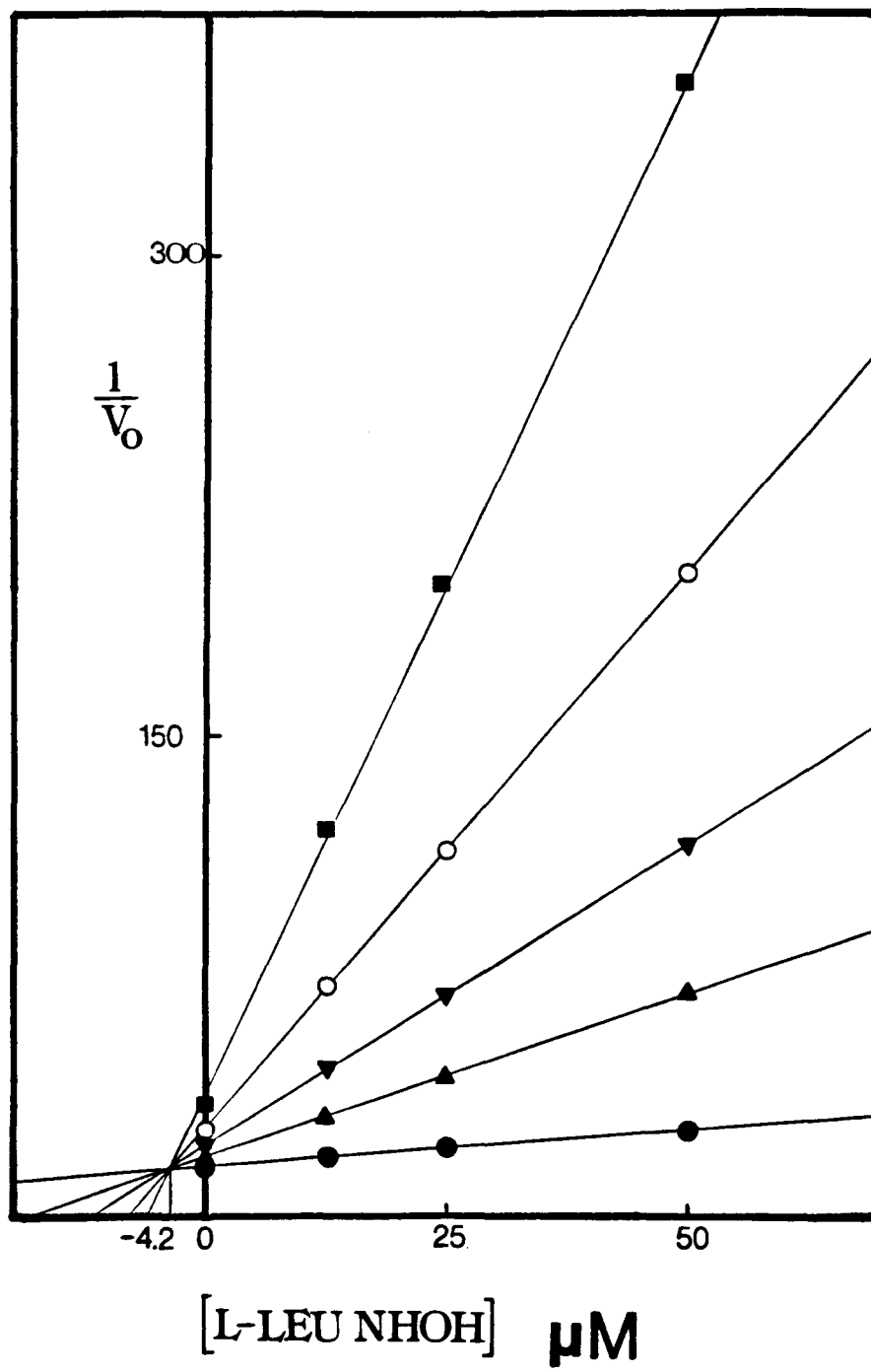


Figure 7- Yonetani-Theorell plot of L-leucine hydroxamate vs. ammonia, under standard conditions. [LeupNA] = 1.0 mM. Ammonium chloride concentrations: ●,0 mM; ▲,25 mM; ▼,50 mM; ○,100 mM; ■,200 mM. Initial velocity is in units of ΔA/min.

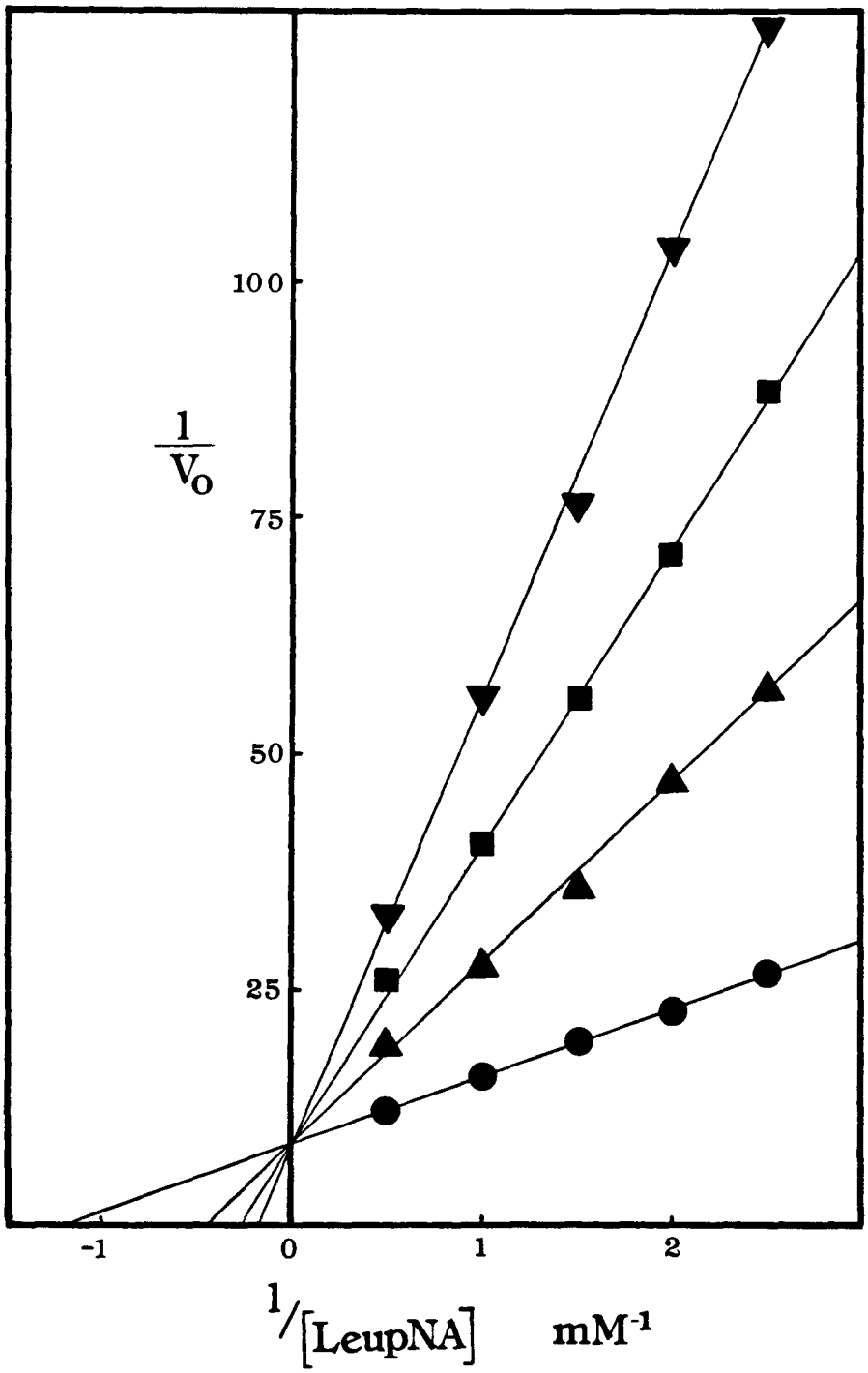


Figure 8- Lineweaver-Burk plot of MAP inhibition by ammonia, under standard conditions. Ammonia (NH_4Cl) concentrations: ●,0 M; ▲,0.1 M; ■,0.2 M; ▼,0.3 M. Initial velocity is in units of △A/min.

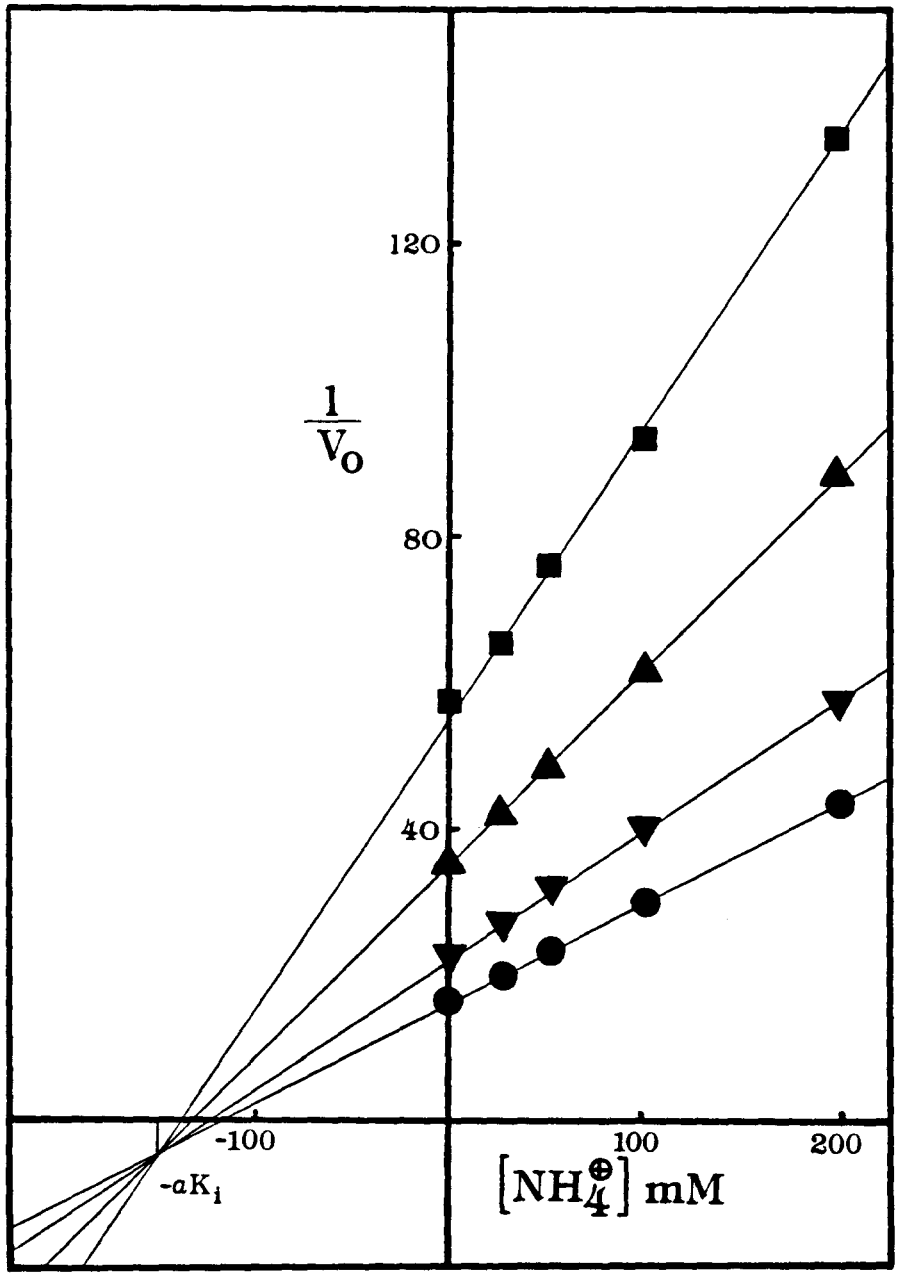


Figure 9- Yonetani-Theorell plot of ammonia vs. D-Leucine hydroxamate, under standard conditions. [LeupNA] = 0.8 mM. D-Leucine hydroxamate concentrations: ●,0 μM; ▼,80 μM;▲,240 μM;■,480 μM. Initial velocity is in units of △A/min.

n-Caproic hydroxamate, glycine hydroxamate and L-leucine hydroxamate (Figure 7) all exhibited the same degree of synergism with ammonia. D-leucine hydroxamate, on the other hand, displayed slightly negative interactions against ammonia (Figure 9). Isoamylamine and ethylamine both bound mutually exclusively against ammonia, indicating a competition for an amine-binding subsite(s). In all cases studied, the amino group of amino acid hydroxamates did not seem to be bound in the same manner as with substrates.

3.2.2 Binding of the Hydrophobic Side Chain

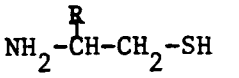
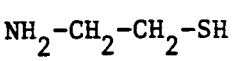
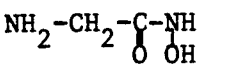
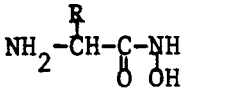
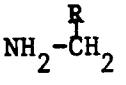
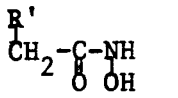
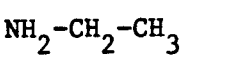
Unfortunately, a good marker for the hydrophobic pocket of the active site was not available. Isoamyl alcohol would have been useful as such except that its high K_i and low miscibility in aqueous buffers made it unpractical as a subsite marker inhibitor. Therefore, isoamylamine (IAA) was chosen as the marker for this subsite. Since the amino group of this inhibitor also binds to the amine-recognition subsite(s), this makes the inhibitor difficult to use as a marker solely for the hydrophobic pocket. A careful interpretation of the Yonetani-Theorell data involving this compound is required (Table VII).

The mercaptoamines, L-leucinthiol and β-MEA appeared to bind almost independently of IAA, or with very slight interference (Figure 10). This indicates the presence of two hydrophobic pockets in the active site, since the alkyl chains of both IAA and L-leucinthiol are bound at once. Similarly, the presence of two separate amine-binding subsites is suggested.

L-leucine hydroxamate exhibited repulsive interactions with IAA

TABLE VII

Binding of the Hydrophobic Side Chain

First Inhibitor		Second Inhibitor	α (±S.D.)	Interpretation[a]
	(L)		1.4	-/I
			1.4 (± 14%)	-/I
			0.48 (± 17%)	+
	(D)		5.0	-
	(L)		3.9	-
			0.62	+
			∞[b]	X

R = $-CH_2-CH(CH_3)_2$ R' = $-(CH_2)_3CH_3$

S.D. is standard deviation.
For assay conditions refer to METHODS.

[a] X = mutually exclusive, - = interference, + = synergism, I = independent.

[b] The upper detection limit for α is approximately ten.

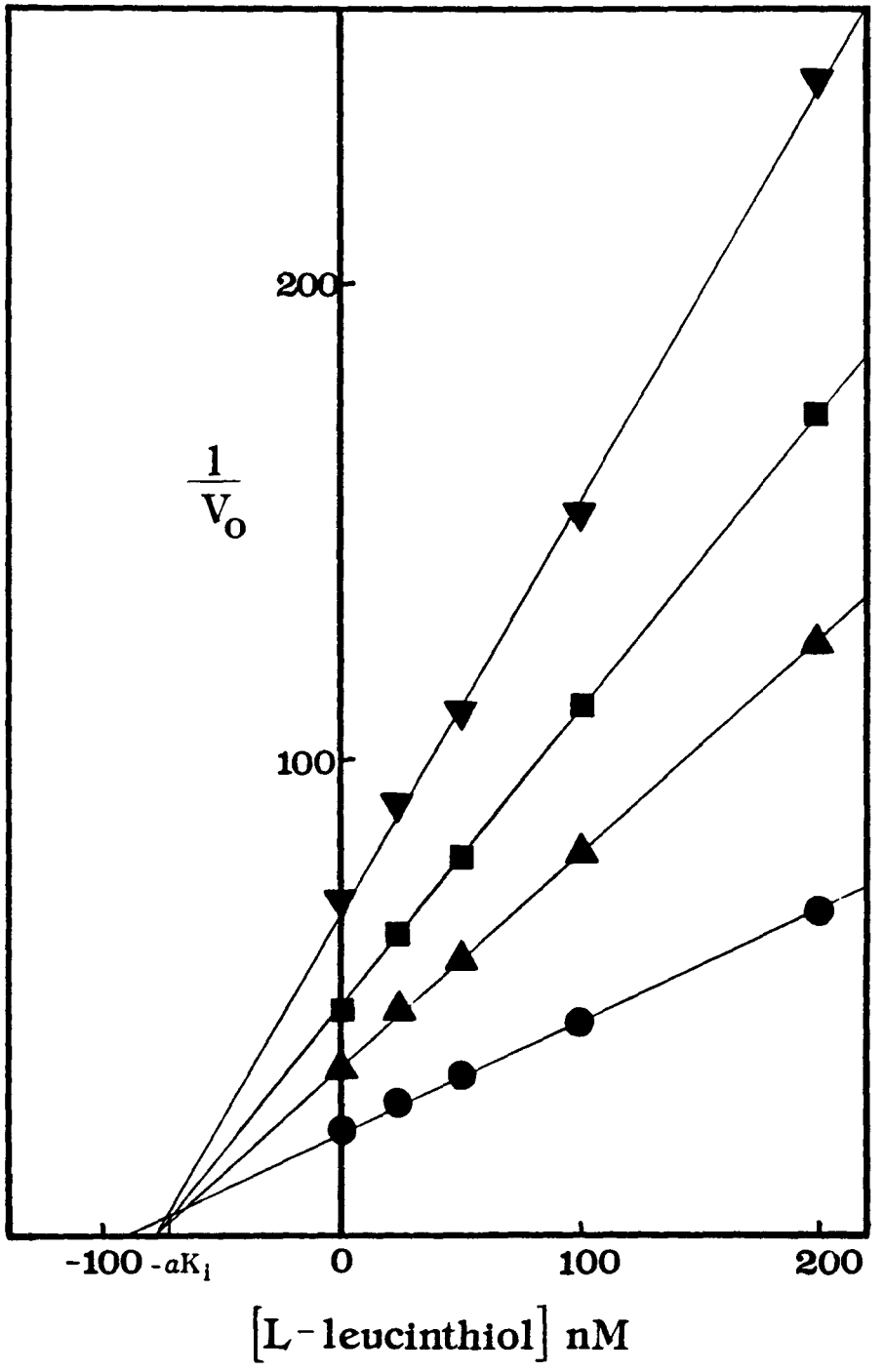


Figure 10- Yonetani-Theorell plot of L-Leucinthiol vs. iso-amylamine, under standard conditions. [LeupNA] = 1.0 mM, [DTT] = 1.0 mM. IAA concentrations: ●,0 mM; ▲,50 mM; ■,100 mM; ▼,200 mM. Initial velocity is in units of ΔA/min.

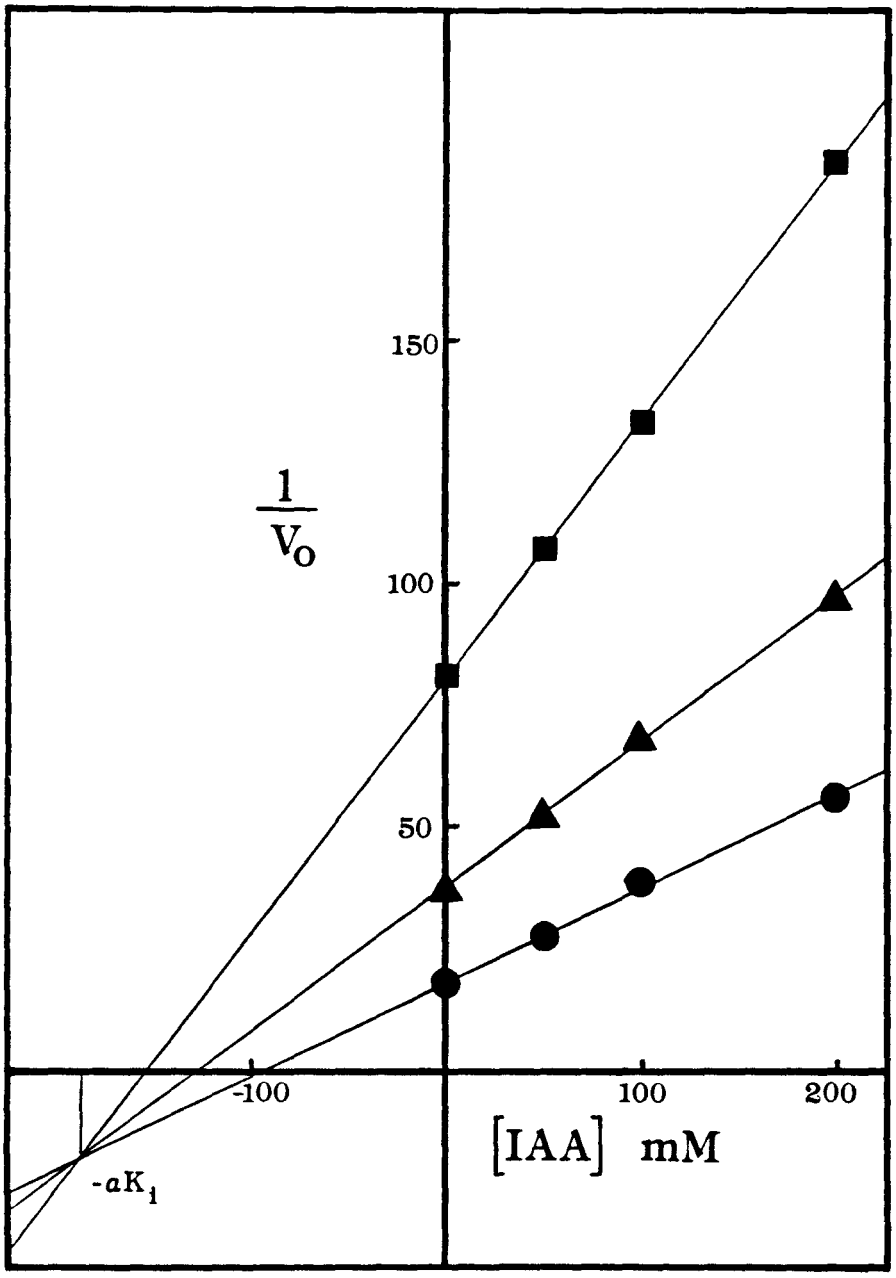


Figure 11- Yonetani-Theorell plot of L-Leucine hydroxamate vs. isoamylamine, under standard conditions. [LeupNA] = 0.8 mM. L-Leucine hydroxamate concentrations: ●,0 μM; ▲,50 μM; ■,150 μM. Initial velocity is in units of △A/min.

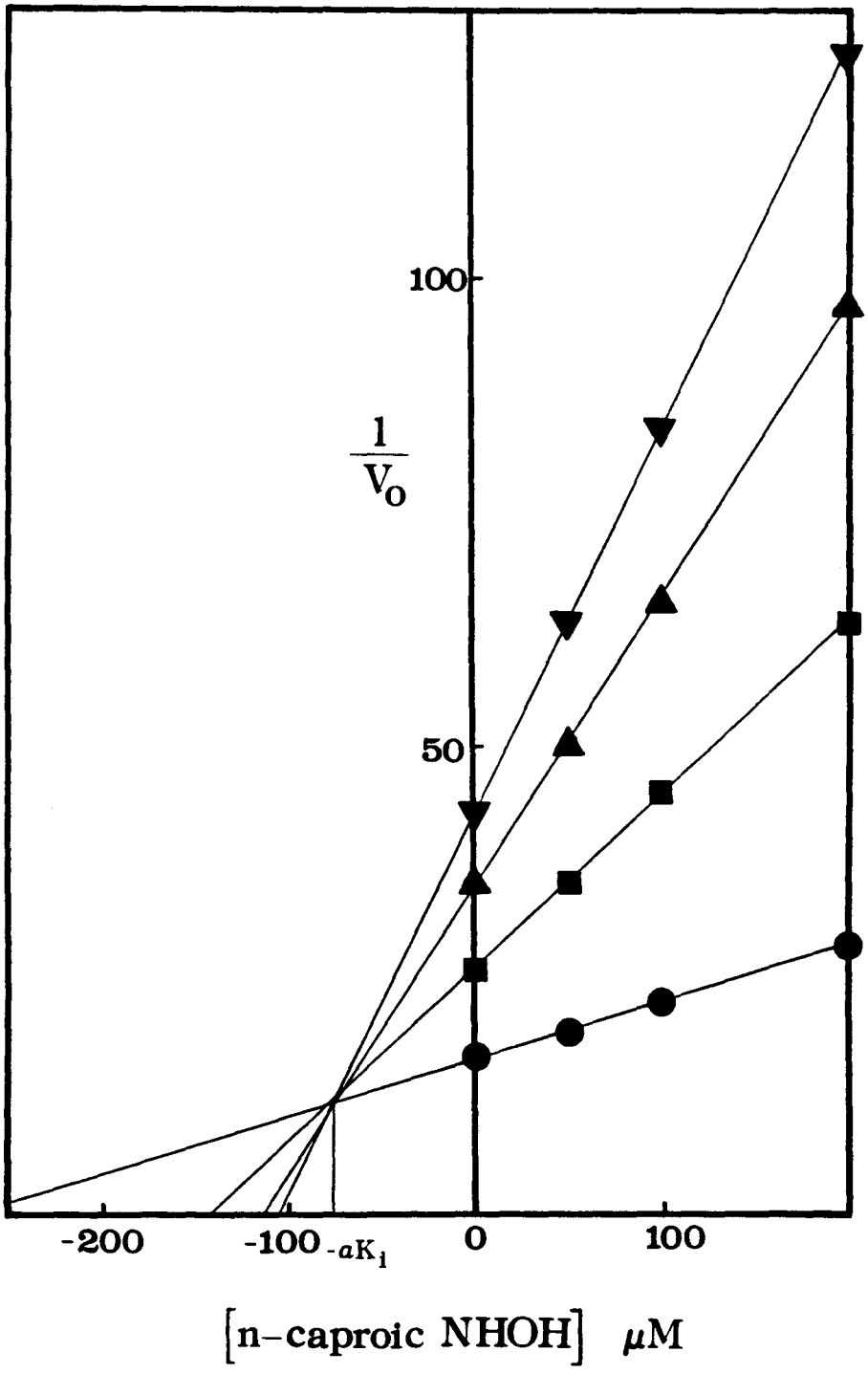


Figure 12- Yonetani-Theorell plot of n-caproic hydroxamate vs. isoamylamine, under standard conditions. [LeupNA] = 1.0 mM. IAA concentrations: ●,0 mM;■,50 mM;▲,100 mM;▼,150 mM. Initial velocity is in units of△A/min.

(Figure 11). The D isomer behaved similarly, having a slightly greater negative interaction. Binding was not mutually exclusive for either hydroxamate, supporting the notion that two distinct hydrophobic pockets are present. Because glycine hydroxamate and n-caproic hydroxamate (Figure 12) both demonstrated synergism with IAA, the amine group of IAA cannot be co-ordinated to the zinc, which would be blocked completely by the bidentate hydroxamate ligand.

Ethylamine, like ammonia, exhibited mutually exclusive binding with IAA.

3.2.3 Binding of Zinc Liganding Groups

Acetohydroxamate was used as the marker for the zinc co-ordination subsite of the active site. All the hydroxamates and mercaptoamines tested were shown to interact at this subsite (Table VIII). The synergistic effect was seen between binding to the zinc and binding to an amine-recognition subsite within the active site, since ammonia and acetohydroxamate displayed positive interactions (Figure 13). This effect was also seen in the cases: ammonia vs. n-caproic hydroxamate, ammonia vs. L-leucine hydroxamate, IAA vs. glycine hydroxamate, IAA vs. L-leucine hydroxamate, ethylamine vs. aceto-hydroxamate and IAA vs. acetohydroxamate. However, in these instances it was unclear whether it was the amine-binding subsite or the hydrophobic pocket which showed the synergistic effect with the zinc ligand.

TABLE VIII

Binding of Liganding Groups

First Inhibitor		Second Inhibitor	α	Interpretation[a]
$NH_2-\underset{R}{CH}-CH_2-SH$	(L)		∞[b]	X
	(D)		∞	X
$NH_2-CH_2-CH_2-SH$			∞	X
$NH_2-\underset{R}{CH_2}$			0.36	+
$NH_2-CH_2-CH_3$		$CH_3-\overset{O}{\overset{\|}{C}}-\underset{OH}{\underset{\|}{NH}}$	0.37	+
NH_3			0.31	+
$\underset{CH_2-\overset{O}{\overset{\|}{C}}-\underset{OH}{\underset{\|}{NH}}}{\overset{R'}{\overset{\|}{}}}$			∞	X
$NH_2-\underset{\underset{O}{\overset{\|}{C}}-\underset{OH}{\underset{\|}{NH}}}{\overset{R}{\overset{\|}{CH}}}$	(L)		∞	X
	(D)		∞	X

$R = -CH_2-CH(CH_3)_2 \quad R' = -(CH_2)_3CH_3$

For assay conditions refer to METHODS.

[a] X = mutually exclusive, + = synergism.

[b] The upper detection limit for α is approximately ten.

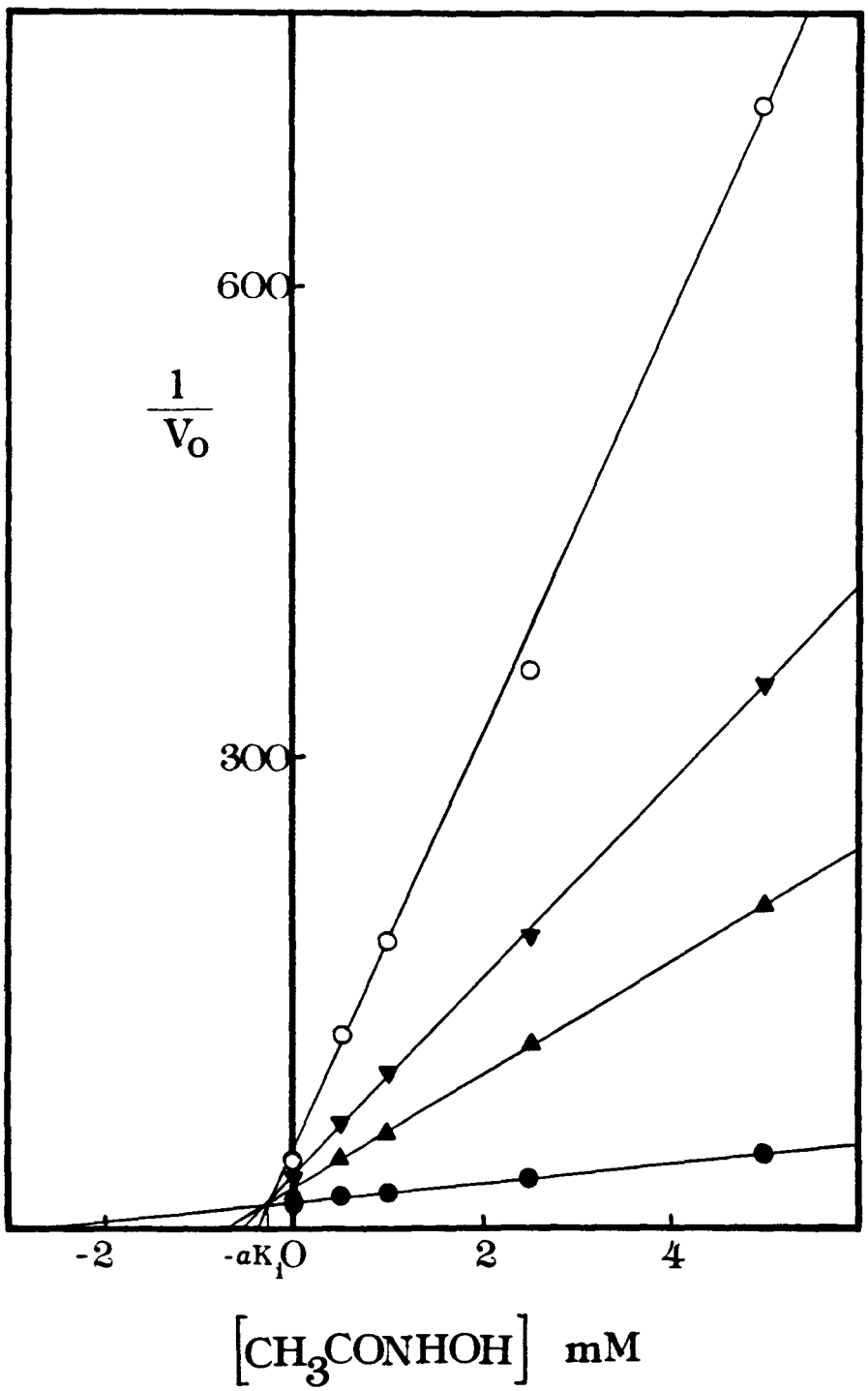


Figure 13- Yonetani-Theorell plot of ammonia vs. acetohydroxamate, under standard conditions. [LeupNA] = 1.0 mM. Ammonia (NH_4Cl) concentrations: ●,0 mM; ▲,50 mM; ▼, 100 mM; ○,200 mM. Initial velocity is in units of ΔA/min.

3.3 EDTA Inactivation Kinetic Studies

3.3.1 Protection of MAP by β-Mercaptoethylamine

It had previously been shown that L-leucine hydroxamate and L-leucinthiol both protect MAP from EDTA inactivation and that the protection constants, K_p, were the same as the K_i values determined for the inhibitors (Chan et al., 1982; Chan, 1983). Initial experiments (performed by D.K.K. Chong) had indicated that β-MEA did not protect from EDTA inactivation. Upon closer examination, using β-MEA concentrations several-fold higher than its K_i of 5.4 μM (at 37°C) (Figure 14), protection was observed (Figure 15) with K_p = 20 μM. Since K_p does not equal K_i, this suggests that two different binding modes exist for β-MEA, only inhibitor molecules which are bound in the lower affinity mode protecting against inactivation.

3.3.2 Protection of MAP by Acetohydroxamate

Acetohydroxamate was also seen to protect MAP from EDTA inactivation, with K_p = 1.2 mM (Figure 16). The K_i of this compound was estimated from a Dixon plot to be 1.3 mM (at 37°C). Thus, for acetohydroxamate, the EDTA inactivation constant (K_p) is approximately equal to the K_i of this compound, implying the existence of only one binding mode for the inhibitor.

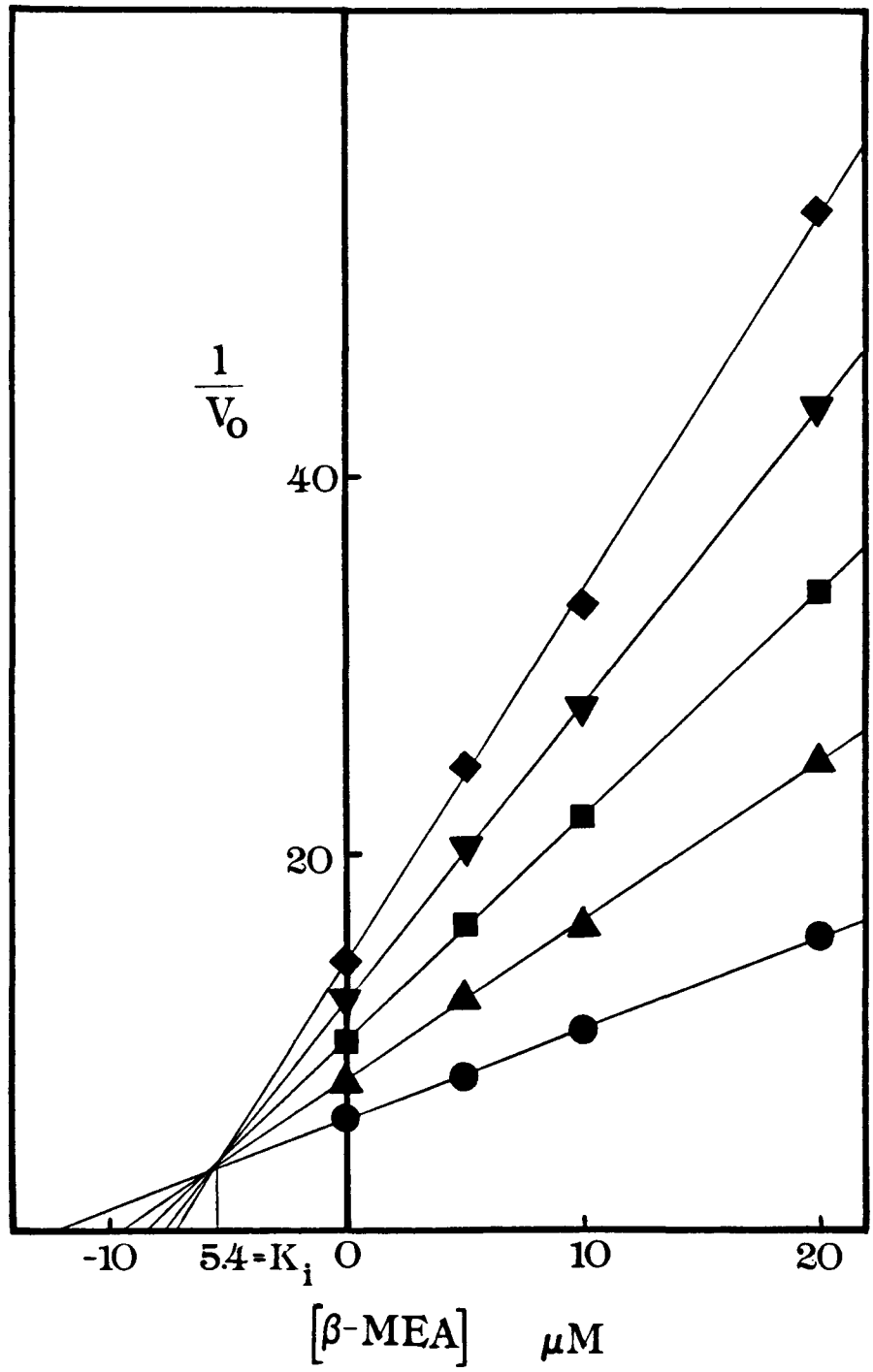


Figure 14- Dixon plot of MAP inhibition by β-mercaptoethylamine at 37°C. [DTT] = 1 mM. LeupNA concentrations: ●,2.0 mM; ▲,1.0 mM; ■,0.67 mM; ▼,0.5 mM; ◆,0.4 mM. Initial velocity is in units of △A/min.

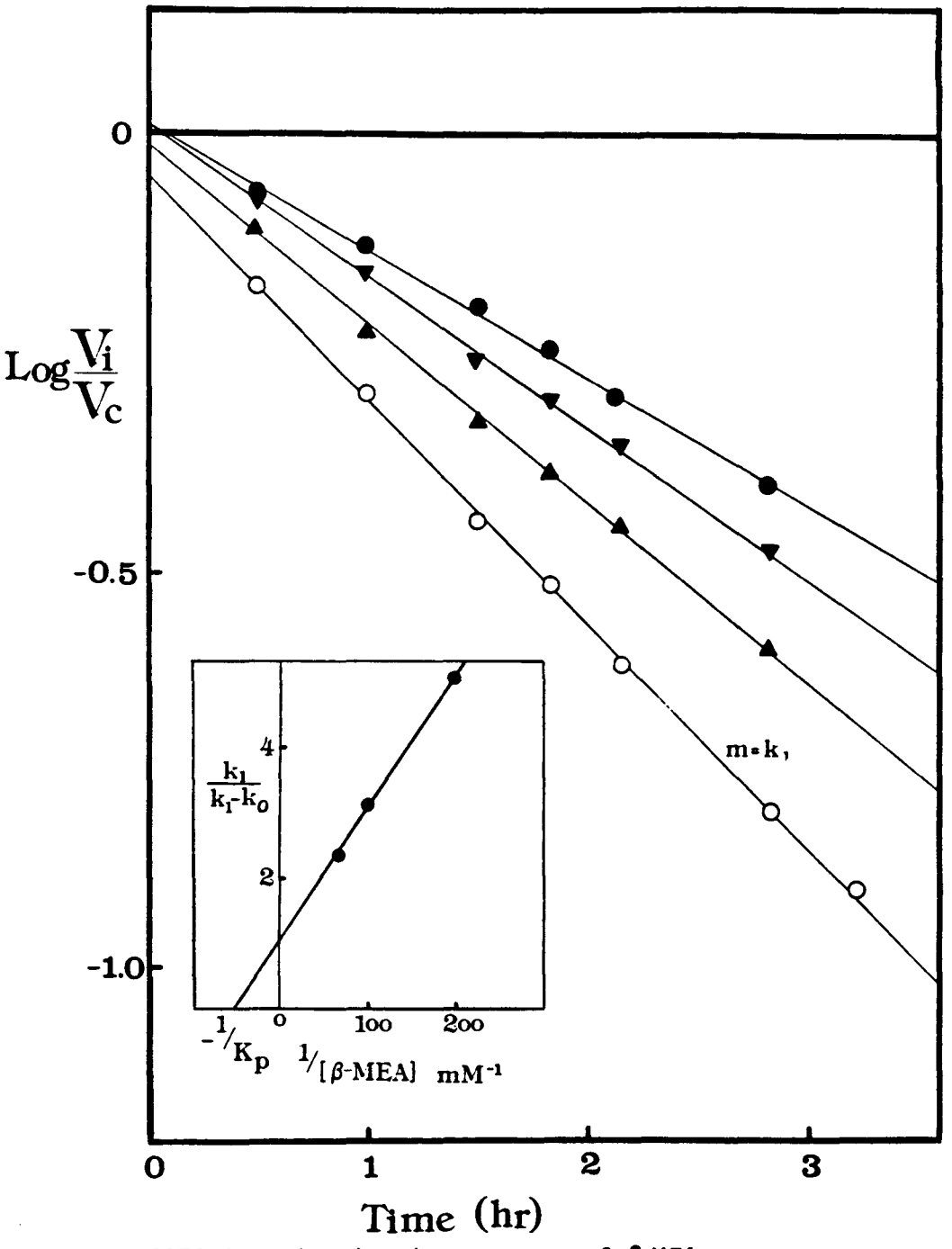


Figure 15- EDTA inactivation in presence of β-MEA. [EDTA] = 6mM, [DTT] = 0.5 mM (in incubation solutions). β-MEA concentrations: ○,0 μM; ▲,5 μM; ▼,10 μM; ●,15 μM. MAP is assayed under standard conditions in 1.0 mM LeupNA.

Inset: K_p determination. k_1=control inactivation rate, k_o = observed inactivation rate with inhibitor. Initial velocity is in units of ΔA/min.

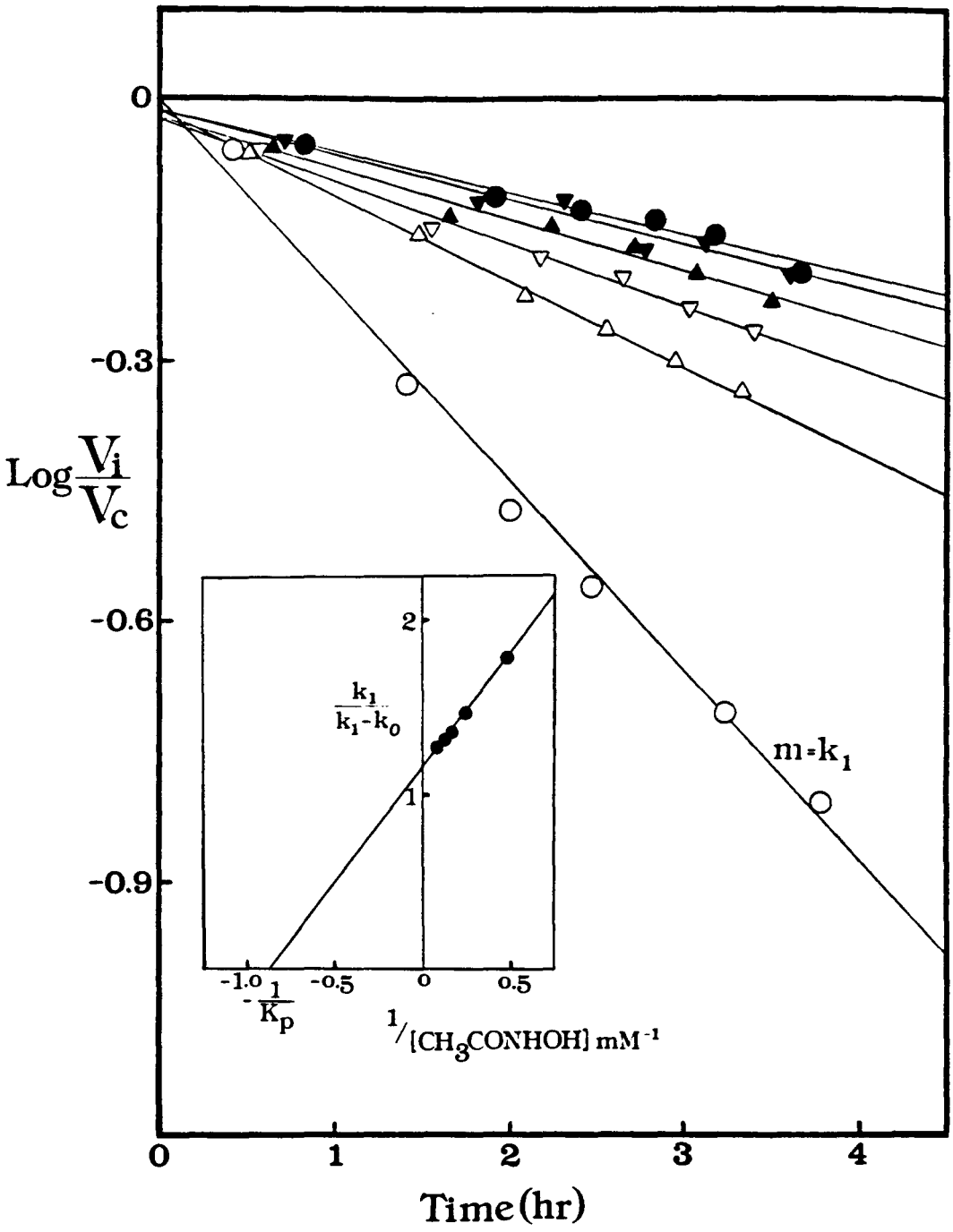


Figure 16- EDTA inactivation in presence of acetohydroxamate. [EDTA] = 6 mM. Acetohydroxamate concentrations: ○,0 mM; △,2.1 mM; ▽,4.2 mM; ▲,6.3 mM; ▼,8.3 mM; ●,12.5 mM. MAP is assayed under standard conditions in 1.0 mM LeupNA.

Inset: K_p determination. k_1 = control inactivation rate, k_o = observed rate with inhibitor. Initial velocity is in units of △A/min.

4. DISCUSSION

4.1 Amines as MAP Inhibitors

The specificity of MAP as an aminopeptidase requires that substrates and inhibitors contain a primary amino group in order to have tight binding to the enzyme. The pK_a of the amino group is an important factor when considering inhibitor potency. A rough correlation seems to exist between pK_a and K_i (Table II), since one decreases with the other. An increase of almost one order of magnitude in potency is seen between methylamine and ammonia, corresponding to a difference in pK_a of 1.37 units between the two. Since only a 2-fold difference in K_i is seen between methylamine and ethylamine (which have virtually identical pK_as) it is unlikely that contributions to binding occurring from short alkyl chains are significant. However, extension of the chain size to that of isoamylamine does give an increase in binding of more than 10-fold. Though IAA has a pK_a 1.37 units larger than ammonia, binding of the hydrophobic chain of the former compensates for this pK_a difference resulting in very similar K_i values for the two compounds.

Free energy calculations comparing the incremental Gibbs energy of transfer from n-octanol to water indicate an increase in the energy of transfer of approximately 2.7 kcal/mole upon addition of a 4-carbon chain to a molecule (Fersht, 1977). This would correspond to approximately a 7-fold increase in binding to the enzyme by the 4-carbon chain

compound compared to the same structure without the alkyl chain. This agrees favourably with the increase in binding seen between methylamine and isoamylamine (10-fold) and supports the idea that the poor pk_a value of IAA is compensated for by binding of the alkyl chain.

An increase in potency of 13-fold is seen between ethylamine and ethylenediamine. This could be explained by the presence in the active site of a second amine-binding subsite, distinct from that involved in substrate recognition. Alternatively, the increased binding could be due to the lowering of the pK_a of the second amino group by the first amino group (by ~2.5 pK_a units). The K_i of 1,3-diaminopropane is 5-fold lower than that of methylamine and about 2-fold lower than that of ethylamine even though all the amino groups have the same pK_a value. This hints at the presence of a second amine-binding subsite, resulting in somewhat better binding of 1,3-diaminopropane compared to monoamines with a similar pK_a.

The influence of a second amino group is seen in the comparison of β-alanine to 2,3-diaminopropionic acid, the latter being 68-fold more potent than the former. In this case, the pK_a of the α-amino group is very low (6.63) compared to that of most other amino groups (e.g. the amino acids, which have a range of 9-10 (Sober, 1968)). The moderately good binding of 2,3-diaminopropionic acid could be due to this decrease in amine pK_a. Another explanation for the potency of this compound is that the presence of an α-amino group is needed for proper binding because of steric reasons. Assuming that the carboxylate group is bound as the zinc ligand, as is known to be the case in CPA and THL (Palmer et al., 1982; Holmes and Matthews, 1981), proper binding of

the amine within the active site may necessitate the α position for this group. β-Alanine would be too large to bind optimally to both subsites at once and is thus a poor inhibitor. Though it is difficult to separate effects due to ionisation from those due to steric constraints, it seems that greater potency may be developed by decreasing the pK_a of the amino group of inhibitors.

4.2 Mercaptoamines as MAP Inhibitors

The importance of the group acting as the zinc ligand is shown in Table III. An increase in potency ranging over one million-fold is seen between the absence of a liganding group (IAA) and the presence of one (L-leucinthiol). The ordering of the potencies of the zinc ligands may be in part determined by the varying effects of the ligand groups on the pK_a of the α-amino group in addition to the differing affinities of the ligands themselves for zinc.

The potency of the mercapto moiety as a zinc ligand together with the necessity of having an amino group present led to the decision to explore mercaptoamines as inhibitors of MAP. Because of the potency of β-mercaptoethylamine, this was chosen as the parent molecular structure of which derivatives were tested as MAP inhibitors (Table V). N-alkylation of the amino group of mercaptoamines results in large decreases in potency reflecting the need for a primary amino group for binding of inhibitors. N-alkylation increases the pK_a of the amino group which has been shown above to be unfavourable to binding. Substitution on the amino group may also give rise to steric hindrances to binding.

The optimal distance between the mercapto moiety and amine group for binding of these compounds is three bond lengths (N-C-C-S). This distance requirement is crucial since γ-mercaptopropylamine, which has a four bond length distance between N and S, is 1,000-fold less strongly bound than β-MEA. This decrease in potency upon extension of the chain size could be attributed to steric hindrance effects or to changes in the pK_a values of either the amino group or thiol group (or both). There is sufficient flexibility in the methylene chain of γ-mercaptopropylamine such that by rotations of the C-C bonds, the amino and thiol groups should be able to occupy positions in space within the active site similar to those of β-MEA. One would not expect that the active site is so rigid in conformation that the extra bulk of one additional methylene group causes such a large decrease in binding. Steric hindrance then is a less likely explanation for the decrease in binding than changes in the pK_a values of the ionisable thiol and/or amino groups. An increase in the pK_a of the mercapto group can be expected upon extension of the alkyl chain length since the inductive effects of the electron-withdrawing amino group will be drastically decreased upon increasing the distance between the nitrogen and sulphur atoms by one carbon atom. The reduced electron-withdrawing effects felt by the thiol results in increased basicity (i.e. an increased pK_a) as compared to β-MEA which has a shorter chain length.

In X-ray diffraction studies on thermolysin (Monzingo and Matthews, 1982) the mercaptan inhibitor 2-benzyl-3-mercapto-propanoyl-L-alanylglycinamide was shown to have the sulphur atom tetrahedrally coordinated in the ionised form to the zinc. The ionised form of thiol

compounds has also been shown to be the bound species for CPA inhibitors (Holmquist and Vallee, 1979). Assuming that the ionised form is also the bound species for MAP, increases in the pK_a of this group could be expected to have unfavourable effects on the K_i. The lower pK_a of the selenol group as compared to the thiol group is likely responsible for the increased inhibition provided by the replacement of sulphur by selenium in mercaptoamines (Table III). At the assay pH of 7.5, virtually all of the selenol group is in the ionised form (pK_a = 4.90; Martell and Smith, 1982), while only about 16 percent of the thiol is in the ionised form (pK_a = 8.21; Martell and Smith, 1982). Ionisation effects are therefore a more plausible explanation than steric constraints for the decreased binding of γ-mercaptopropylamine as compared to β-MEA.

The presence of a second amino group on the γ-carbon (i.e. 1,3-diamino-2-mercaptopropane) was clearly unfavourable (Table V) by a factor of fifty-three. This may be due to steric hindrance effects, electrostatic repulsions or a combination of both. The observation that (D,L)-2,3-diaminopropionic acid is 68-fold more potent than β-alanine (Table II) led to the postulated existence of a second amine-binding subsite in addition to the subsite responsible for recognition of the substrate α-amino group. Unfortunately, this idea was unsupported due to the failure of 1,3-diamino-2-mercaptopropane to be a good MAP inhibitor. However, an amino group in the β position (i.e. 2,3-diaminopropanethiol) may be favourable to binding since this structure is analogous to (D,L)-2,3-diaminopropionic acid (both containing vicinal amino groups). The potency of the inhibition of MAP by this

compound would help to shed light on the existence of a second amine-binding subsite but unfortunately it was not available for testing.

The inclusion of a large, hydrophobic substituent on the α-carbon gives an increase in binding of almost two orders of magnitude which is similar to the hydrophobic binding effect seen in Table II as well as that seen for the amino acid hydroxamates (Chan et al., 1982). L-stereochemistry is required for the α substitution, D-stereochemistry being about 1,000-fold less effective. This stereospecificity mirrors that observed for substrates since D amino acids are not hydrolysed from peptides. The stereospecificity shown towards L-leucinthiol then supports the notion that mercaptoamines bind in the same manner as do substrates. Further, the high potency of L-leucinthiol indicates that it is likely a transition state analogue inhibitor.

4.2.1 Specificity of Mercaptoamines towards MAP

The comparison between three zinc proteases (i.e. carboxypeptidase A, thermolysin and microsomal aminopeptidase) of the potencies of inhibition of the β-MEA derivatives illustrates their relative specificity as MAP inhibitors (Table V). These compounds are least potent with CPA which requires a carboxyl group for binding of inhibitors and substrates. The positively charged amino group of mercaptoamines would likely give rise to repulsions with the similarly charged Arg-145 residue present in the active site of CPA (Kester and Matthews, 1977). THL, being an endopeptidase, has no requirements for either a free amino group or carboxyl group such that in the active site there are neither repulsive nor attractive interactions for the

amino group of mercaptoamines, the net result being that binding of mercaptoamine inhibitors to THL is intermediate in strength between MAP and CPA. The generally noncompetitive nature of the inhibition of CPA and THL by these compounds, compared to the completely competitive inhibition of MAP, is also indicative of their specificity towards MAP. The fact that γ-mercaptopropylamine is a somewhat better inhibitor of CPA than of MAP may be due to the larger size of this molecule compared to β-MEA, allowing some interaction of the amino group at a site within CPA which is not attainable by β-MEA nor its derivatives. D- and L-leucinthiol have similar K_i values with CPA which is also consistent with the lack of an amine-binding subsite in the enzyme. Only the side chain and thiol groups would likely be bound in a hydrophobic pocket and at the zinc, respectively. The difference in K_i between the two isomers is only 9-fold for THL which indicates the absence of an amine-binding subsite in THL. D-leucinthiol is in fact a slightly better (7-fold) inhibitor of THL than of MAP where binding of the amino group and thiol group impose stereospecificity with respect to binding of the alkyl chain of the molecule. In the cases of CPA and THL, binding of the alkyl chain and thiol would impose a stereospecificity with respect to binding of the amino group if a binding subsite were present for it. Presumably, the amino group does not create appreciable steric hindrances to the binding of either isomer to CPA or THL because of its small size. The apparent specificity of L-leucinthiol towards MAP would be yet greater except for the presence of the hydrophobic pockets in the active sites of CPA and THL which can bind the alkyl chain of leucinthiol.

4.3 MAP Subsites

Thus far, three distinct types of subsites are clearly present within the active site of MAP: amine-binding, hydrophobic pocket and zinc coordination subsites. The relative importance of these three subsites to inhibitor binding can be empirically ranked as: zinc ligand > amine $\geq$ hydrophobic pocket.

Compounds such as isoamylamine, which lack a zinc ligand, or as L-leucinol, which possess only a weak ligand, are very poor inhibitors (Table III) and are several orders of magnitude less potent than L-leucinthiol. This testifies as to the importance of this binding subsite. The presence of a hydrophobic group in an inhibitor molecule typically increases the potency one to two orders of magnitude (e.g. β-MEA vs. L-leucinthiol, Table V). The presence of an α-amino group increases binding only a few fold for some inhibitors such as the amino acid hydroxamates (e.g. n-caproic hydroxamate vs. (D,L)-norleucine hydroxamate and glycine hydroxamate vs. acetohydroxamate, Table IV). However, for the mercaptoamines the presence of an amino group increases potency by three or more orders of magnitude since β-mercaptoethanol has a K_i in the millimolar concentration range (D.S. Pickering, unpublished results) while β-MEA has a K_i in the micromolar range. This illustrates the importance of the amino group to the binding of mercaptoamines. These differences in amino group binding suggest a difference in the mode of binding of mercaptoamines and amino acid hydroxamates with respect to the amino group. Also, the stereoselectivity shown by MAP against mercaptoamines is on the order of 1,000-fold (Table V) while it is only

2-fold for the hydroxamates (Table IV). It is evident therefore that the binding modes of these two classes of inhibitor are different.

4.3.1 Amine-Binding Subsites

The results of multiple inhibition studies indicate that two amine-binding subsites are present since the amino group of mercaptoamines is observed to bind competitively against ammonia while the amino acid hydroxamates do not (Table VI). Also, the amino group of D- and L-leucine hydroxamate is seen to bind differently with respect to ammonia (Table VI). L-leucine hydroxamate exhibits synergistic interactions while the D isomer shows slight interference to ammonia binding. Ammonia is a strictly competitive inhibitor of MAP (Figure 8) with a relatively small K_i (58 mM) considering the simple structure of the molecule. It is therefore reasonable to assume that the binding subsite for ammonia is the same one responsible for recognition of the substrate amino group.

Additional evidence for two distinct amine-binding subsites comes from the ability of IAA and L-leucinthiol to bind to the active site simultaneously and independently (Table VII). IAA and β-MEA also bind simultaneously and independently while L- and D-leucine hydroxamate bind simultaneously against IAA with moderately negative interactions. These data imply the existence of at least two amine-binding subsites. One of these subsites (hereafter designated A1) is the subsite responsible for binding of the N-terminal amino group of substrates and which gives rise to the enzyme's classification as an aminopeptidase. It seems to be this subsite which exhibits the

synergistic effect with the zinc coordination subsite, as demonstrated by the multiple inhibition studies of ammonia vs. acetohydroxamate (Table VIII) where synergism is seen. The second amine-binding subsite (A2) may be an active site residue which has positive interactions with the nascent amino group following hydrolysis of the scissile bond.

It had been proposed (Bryce and Rabin, 1964) that the amino group of substrates and inhibitors may actually ligand the zinc ion in cytosolic aminopeptidase. In the case of the amino acid hydroxamates however, the amino group is not likely to be a zinc ligand since the bidentate hydroxamate moiety should block this subsite completely. Moreover, the ability of IAA to bind in a synergistic fashion with acetohydroxamate again would appear to proscribe binding of the amino group of this inhibitor at the zinc coordination subsite.

In one proposed hydrolytic mechanism for CPA the active site residue Tyr-248 forms a hydrogen bond with the NH of the scissile bond, eventually donating a proton to the nitrogen upon formation of the nascent amino group (Lipscomb, 1980). With this precedent in mind it is proposed that some residue may be present in the active site of MAP which serves a similar function.

4.3.2 Hydrophobic Pocket Subsites

The poor potency of α-aminoisobutyric hydroxamate (Table IV) indicates that the orientation of the hydrophobic pocket binding the ultimate N-terminal residue of substrates is regiospecific with respect to the amino and hydroxamate groups, since the hydrophobic pocket cannot accommodate both methyl groups simultaneously. Only one of the methyl groups is properly oriented to fit into the pocket, the second methyl group being directed away from this pocket and creating steric interferences to binding. Therefore, the pocket does not partially encircle substrate or inhibitor molecules about the plane of the R-C-H bonds, where R represents the amino acid side chain.

Multiple inhibition studies of isoamylamine vs. mercaptoamines (Table VII) indicate independent binding between these inhibitors. Two distinct hydrophobic pockets must therefore be present. It has been shown in the case of polyalanine substrates of cytosolic aminopeptidase that residues further removed from the N-terminus have an influence on the rate of hydrolysis of the peptide, indicating the presence of additional hydrophobic pockets (Schechter and Berger, 1966). Similar findings have also been reported for CPA (Abramowitz et al., 1967). In analogy, one may expect that the side chain of the N-terminal penultimate residue of substrates and inhibitors will also affect their binding to MAP. The second hydrophobic pocket (S2) observed in multiple inhibition studies is then likely the penultimate amino acid side-chain-binding subsite for substrates while subsite S1 is the hydrophobic pocket which binds the side chain of the N-terminal residue (Figure 17).

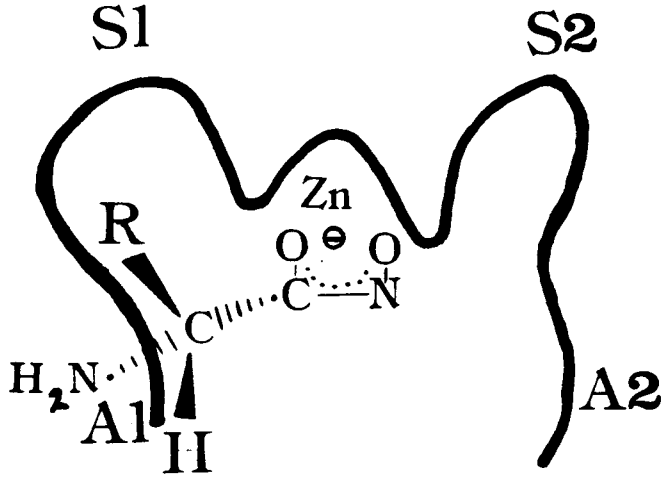
4.4 Binding of Mercaptoamine and Hydroxamate Inhibitors

4.4.1 Leucinthiol Binding

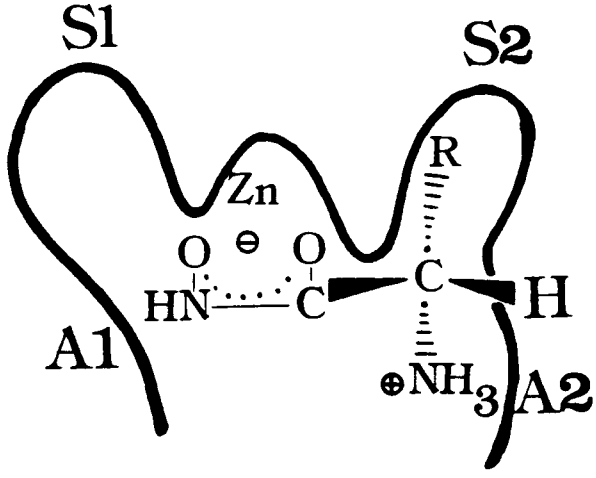
From the high potency and stereospecificity of L-leucinthiol binding, it was proposed that this compound binds in the same mode as substrate, acting as a transition state analogue inhibitor. The stereoselectivity shown resembles that found for substrates, while the tetrahedral geometry about the β-carbon with the thiol substituent would resemble that of the proposed transition state (Figure 6). The multiple inhibition studies against ammonia (Table VI) indicate that the amino group of L-leucinthiol is bound within the enzyme's active site. The importance of the amino group to the binding of mercaptoamines parallels the necessity of substrates having a primary amino group. This substrate-like amino group recognition supports the transition state analogue nature of mercaptoamine inhibitors.

Under the assumption that L-leucinthiol binds in a substrate-like fashion, it is proposed that the leucyl side chain of this inhibitor binds to subsite S1. Simultaneous occupancy of subsite S2 by IAA is permitted without interference since IAA does not ligand to the zinc (as indicated in Table VIII by its synergistic binding against acetohydroxamate, glycine hydroxamate and n-caproic hydroxamate). Binding of IAA then does not interfere with the coordination of the thiol group of mercaptoamines to the zinc.

In the case of the binding of D-leucinthiol to MAP, there are several possibilities available. Most importantly, if there is a very small (0.14% or less) contamination by the L isomer, the true



D-Leucine Hydroxamate



L-Leucine Hydroxamate

Figure 17- Active site model of MAP.
S1 and S2 represent the ultimate and penultimate amino acid side chain binding sites for peptide substrates. A1 represents the substrate amine-binding site while A2 represents the site which is proposed to bind the nascent amino group upon substrate hydrolysis. L-Leucinthiol is proposed to bind at sites S1, A1 and at the zinc. D- and L-Leucine hydroxamate binding is shown.

inhibition constant of the D isomer may be several orders of magnitude larger than the apparent K_i of 36 μM. The true K_i of the D isomer (K_{iD}) may be calculated from the apparent K_i once the mole fraction contamination by the L isomer ($\bar{L}$) is known:

$$1/K_{i\,(app.)} = \bar{L}/K_{iL} + (1 - \bar{L})/K_{iD}$$

$$K_{iD} = 36\ (1 - \bar{L})/[1 - (36/0.051)\bar{L}]$$

$$= 36\ (1 - \bar{L})/(1 - 705.9\bar{L})\ (\mu M)$$

L contamination of the D isomer may arise from an impure synthetic reagent (D-leucine methyl ester) used for the preparation of D-leucinthiol (Pickering et al., 1985). In this case, the contamination would carry through all of the synthetic reactions to the final product. Alternatively, racemisation might possibly occur to a minor degree at one or more of the synthetic steps to give some L isomer in the final product. Attempts to determine the percent of L isomer in the starting reagent or final product were unsuccessful. If the inhibition constant for the D isomer is actually larger, this would indicate that the inability of the hydrophobic chain of D-leucinthiol to bind in the active site (since binding of the amino and thiol groups have priority) causes severe steric hindrances to binding of the inhibitor.

On the other hand, if the observed K_i value for D-leucinthiol is the true (or approximate) K_i of the D isomer, then the active site must be able to at least partially accommodate the hydrophobic group in some fashion. The results obtained with α-aminoisobutyric hydroxamate

revealed the regiospecific orientation of the S1 pocket with respect to the methyl substituents of the α-carbon. This implies that binding of the hydrophobic side chain of D-leucinthiol cannot occur at S1 (where L-leucinthiol binds), assuming that the thiol group is coordinated to the zinc and the amino group is bound at subsite A1 as is indicated by the multiple inhibition studies against ammonia (Table VI). One manner in which binding of the side chain may occur would be by 'backwards' binding at subsite S2 with the thiol coordinated to the zinc and the amino group perhaps bound at the second amine-binding subsite, A2. The decrease in potency, as compared to the L isomer, could then be attributable to weak binding of the amino group to subsite A2 and/or to a suboptimal coordination distance or geometry between thiol and zinc. The fact that D-leucinthiol apparently binds mutually exclusively against ammonia (as does L-leucinthiol) suggests that the inhibition observed is due to L isomer contamination.

4.4.2 Leucine Hydroxamate Binding

The lower potency and lack of stereospecificity of L- and D-leucine hydroxamate binding to MAP (compared to L- and D-leucinthiol) suggest that these compounds are product analogue inhibitors. In addition, the trigonal geometry of D- and L-leucine hydroxamate resembles that of the carboxyl group of leucine, a product of substrate hydrolysis. The moderately good binding of acetohydroxamate suggests that the potency of D- and L-leucine hydroxamate is largely due to the postulated bidentate chelation of the active site zinc by the hydroxamate moiety. Also, these two isomers appear to bind differently

according to the K_i and multiple inhibition studies performed.

The small difference (4-fold) in K_i value between n-caproic hydroxamate and (D,L)-norleucine hydroxamate (Table IV) is interpreted to mean that the amino group of hydroxamates does not make a large contribution to the binding of these inhibitors in contrast to the case with mercaptoamines. The multiple inhibition studies of D- and L-leucine hydroxamate versus ammonia (Table VI) indicate that the isomers do not bind in the same mode with respect to the amino groups. The fact that n-caproic hydroxamate induces the same degree of synergism with ammonia as L-leucine hydroxamate, using multiple inhibition kinetics, suggests that they bind in a similar manner. This synergism indicates that the amino group of L-leucine hydroxamate is not bound at the ammonia binding subsite. Glycine hydroxamate also exhibits synergistic binding against ammonia with an α value similar to that for L-leucine hydroxamate and n-caproic hydroxamate. This suggests that the amino group of these amino acid hydroxamates is unbound. D-leucine hydroxamate, on the other hand, shows slight interference against ammonia, indicating that its amino group may be bound to some degree and sterically or electrostatically hinders the binding of ammonia. Therefore, the amino group of D-leucine hydroxamate seems to be near the synergistic amine-binding subsite A1 while the amino group of L-leucine hydroxamate is near the second amine-binding subsite, A2, and may or may not be bound (Figure 17). The bidentate binding mode of the hydroxamate ligand and the presence of one extra bond distance (as compared to the carboxylate ligand) between the amino nitrogen and negative charge of the ligand group, as well as binding of the hydro-

phobic group, may together place constraints upon the positioning of the amino group of D-leucine hydroxamate within the active site of MAP such that it approaches subsite A1 to disrupt the binding of ammonia.

Studies on the variation of inhibitor potency with assay pH (D.S. Pickering, unpublished results) for D- and L-leucine hydroxamate support a difference in binding modes of the two isomers. In general, the potency of the D isomer increases at more acidic pH values while the opposite is true of the L isomer (in the pH range six to nine). The amino group of the D isomer may be bound to the protonated form of some active site residue while the amino group of the L isomer is bound to a basic group. However, the preliminary nature of these results does not allow an unequivocal interpretation.

Since L-leucinthiol occupies both subsites A1 and S1, and D-leucine hydroxamate binds at A1, it is proposed that D-leucine hydroxamate binds to subsites S1 and A1 (Figure 17). L-leucine hydroxamate then, binds to subsite S2 (and A2 perhaps) in a 'backwards' mode as is known to happen in its binding to thermolysin, (Holmes and Matthews, 1981). In this case, ammonia and the other smaller amine inhibitors (which do not interfere at the zinc binding subsite) may still bind to subsites A1 and S1 to produce the synergism observed with L-leucine hydroxamate (Table VI).

Isoamylamine shows small interferences against simultaneous binding of either D- or L-leucine hydroxamate (Table VII). IAA likely is able to bind to either subsite S1 or S2, whichever is not occupied by one of the hydroxamate inhibitors. The mixed-noncompetitive nature of the inhibition by IAA agrees with this notion of two binding modes

being available for the inhibitor. Since n-caproic hydroxamate is synergistic with IAA, it must be the amino group of D- and L-leucine hydroxamate which interferes in some manner with the binding of IAA. Glycine hydroxamate, like acetohydroxamate, binds synergistically with IAA indicating a lack of interference of the amino group to IAA binding. Glycine hydroxamate may only be bound at one point, the zinc coordination subsite while L-leucine hydroxamate would be bound at the zinc coordination and S2 hydrophobic pocket subsites.

The above model supports a product-analogue nature of MAP inhibition by amino acid hydroxamates, by 'backwards' binding of L-leucine hydroxamate and 'normal', substrate-like binding of the D isomer.

4.4.3 β-Mercaptoethylamine Binding

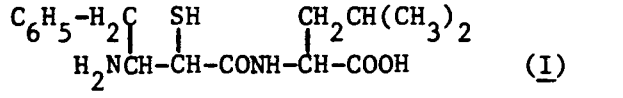
The EDTA inactivation kinetics of β-MEA (Figure 15) indicate that two binding modes are available for this inhibitor since the value of the EDTA-protection constant K_p (20 μM) is different from the K_i at 37°C (5.4 μM). The lower affinity mode is responsible for protection against inactivation while the higher affinity mode is that of the K_i value determined from Linewaver-Burk and Dixon plots and which does not protect the enzyme from EDTA inactivation.

The fact that acetohydroxamate also protects against EDTA inactivation, with a K_p value similar to the K_i, shows that a simple, bidentate chelation of the zinc by the hydroxamate moiety is capable of decreasing the temperature dependent dissociation of zinc from the active site.

4.5 Future Directions to Inhibitor Design

The specificity requirements of MAP inhibitors are met very well by mercaptoamines, in particular by L-leucinthiol. Increased specificity and potency would best be achieved by an expansion of the basic structure of L-leucinthiol in terms of new or better functional groups.

In designing MAP inhibitors of greater potency, consideration should be given to the inclusion of functional groups capable of lowering the pK_a of the α-amino group of the inhibitor while inclusion of a second amino group in the proper position (γ to the thiol) may allow additional binding to the hypothetical subsite A2. Incorporation of another hydrophobic group into the structure of L-leucinthiol, enabling occupancy of subsite S2, is one route to the design of more potent inhibitors. Compounds such as bestatin, amastatin and simple oligopeptide inhibitors make use of this subsite and presumably additional subsites such as S3, S4, etc. The compound thio-bestatin (I) is one example of an



(I)

extended-size mercaptoamine inhibitor which should be an extremely potent inhibitor.

Increased potency may also be achieved by replacement of the thiol group by a selenol group (Table III). Although the increase in potency observed (4-fold) is modest, a larger increase may possibly be

seen in the case of compounds containing hydrophobic side chains, such as L-leucinselenol or seleno-bestatin, where the binding to the hydrophobic pocket by the side chain might allow a stronger coordination of the zinc by the selenol group. Many precedents exist for the replacement of sulphur by selenium to give compounds of increased biological activity, (Shamberger, 1983). This possibility requires further investigation to be either substantiated or invalidated.

4.6 Prospective Analgesics

The significance of these suggested structural modifications to the long term objective of the development of new analgesic drugs cannot as yet be fully assessed. Pending the elucidation of the exact role of brain microsomal aminopeptidase in enkephalin degradation and the characterisation of its active site, the relevance of kidney MAP inhibitors in this scheme is unknown. If, however, the kidney and brain enzymes are one in the same (or are very similar) as the preliminary data indicate, then additional complications are likely to arise upon the *in vivo* application of compounds initially evaluated by their *in vitro* K_i.

The instability of the reduced form of mercaptoamines in body fluids such as blood may be problematic since oxidation of the thiol may occur (Kun, 1961; Cavallini et al., 1961). This potential problem could be circumvented by the design of a pro-drug where the thiol group is left in the form of a thiol ester which slowly hydrolyses to the free thiol *in vivo*. Thus, a slow and constant release of active drug

from the pro-drug pool would occur. The selenol group is superior to the thiol group with respect to chemical stability since its tendency towards oxidation is less. A higher *in vivo* stability can be expected as well since, biologically, selenium tends to be found in the reduced form (Cerwenka and Cooper, 1961). Selenium toxicity may then become a hindrance to the use of these compounds as drugs, though this is less likely to be a worry because of the very low clinical doses which would be needed to be given for a highly potent drug and in view of the fact that organic selenium compounds are generally less toxic than inorganic selenium compounds (Martin, 1973).

Perhaps the most important impediment confronting the use of these compounds as drugs would be the relative distribution *in vivo* of these potential drugs to the different body tissues. Rapid metabolic inactivation and elimination may occur from systemic circulation while the brain is the actual target tissue. These compounds may not be able to penetrate the blood-brain barrier (Van Deurs, 1980) so that their concentration in the brain would be too low to be effective.

There exists two major passageways into the brain for compounds: passive diffusion across the blood-brain barrier and carrier-mediated transport (Bodor, 1985). By simply increasing the hydrophobicity of a compound, passive diffusion through the membranes of the blood-brain barrier increases. Therefore, increasing the lipophilicity of mercaptoamine pro-drugs may help increase analgesic efficacy. Esterification of the thiol and acylation of the amine are the two immediate methods of decreasing the polarity of these compounds. Non-enzymatic (and enzymatic) hydrolysis of the ester and enzymatic

hydrolysis of the amide would then liberate active inhibitor.

In the carrier-mediated transport pathway the pro-drug is covalently coupled to some compound for which transport protein(s) exist to move the substance across the blood-brain barrier. This approach has had success with compounds such as phenylethylamine, dopamine and tryptophan (Bodor and Farag, 1983) and in theory could be similarly useful for MAP inhibitors.

In conclusion, it can be said that although the prospects for increasing the potency of MAP inhibitors are encouraging, many theoretical and practical obstacles, some of which have been outlined above, remain to be solved before the appearance of these potentially analgesic drugs in the pharmacy.

APPENDIX

EDTA Inactivation Kinetics

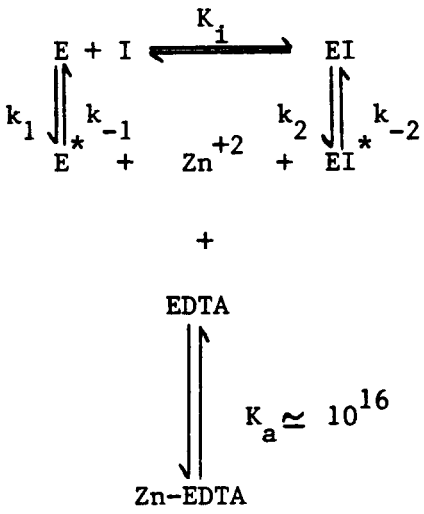


FIGURE 18 - EDTA Inactivation Equilibria for a Postulated Mechanism.

E = active holoenzyme, I = inhibitor
$K_i = [E]\,[I]/[EI]$, E^* = inactive apoenzyme,
EI^* = apoenzyme-inhibitor complex,
K_a = association constant, k_1, k_{-1}, k_2, k_{-2} = unidirectional rate constants.

$[EDTA] \simeq 10$ mM

$$K_a = \frac{[Zn\text{-}EDTA]}{[Zn^{+2}][EDTA]} \simeq 10^{16}$$

$$\frac{[Zn\text{-}EDTA]}{[Zn^{+2}]} \simeq 10^{14} \quad \text{i.e. } [Zn^{+2}]_{free} \simeq 0$$

$$-\frac{d[E]}{dt} = \text{inactivation rate} = k_1[E] - k_{-1}[E^*][Zn^{+2}] + k_2[EI] - k_{-2}[EI^*][Zn^{+2}]$$

$$= k_1[E] + k_2[EI] = k_{obs}[E_{total}]$$

$$= k_1[E] + k_2[EI] = k_{obs}([E] + [EI])$$

$$k_1[E] + k_2[E][I]/K_i = k_{obs}[E] + k_{obs}[E][I]/K_i$$

$$k_1K_i + k_2[I] = k_{obs}K_i + k_{obs}[I]$$

$$k_1K_i - k_{obs}K_i = k_{obs}[I] - k_2[I]$$

$$K_i(k_1 - k_{obs}) = [I](k_{obs} - k_2)$$

$$\frac{k_{obs} - k_2}{k_1 - k_{obs}} = K_i/[I]$$

$$\frac{k_{obs} - k_2 + (k_1 - k_{obs})}{k_1 - k_{obs}} = \frac{K_i}{[I]} + 1$$

$$\frac{k_1 - k_2}{k_1 - k_{obs}} = \frac{K_i}{[I]} + 1$$

$$\frac{1}{k_1 - k_{obs}} = \frac{1}{k_1 - k_2} + \frac{K_i}{k_1 - k_2}\frac{1}{[I]}$$

$$(1) \qquad \frac{k_1}{k_1 - k_{obs}} = \frac{k_1}{k_1 - k_2}\left(1 + \frac{K_i}{[I]}\right)$$

If $1/[I]$ is plotted against $k_1/(k_1 - k_{obs})$, a straight line is obtained with an x-intercept of $-1/K_i = -1/K_p$. This constant, K_p, is a measure of the degree to which an inhibitor prevents dissociation of zinc ion from the holoenzyme. K_p is the dissociation constant for that EI complex which hinders the removal of zinc from the active site. If more than one binding mode exists, then the observed K_i may not be the same as K_p, (i.e. $1/K_{obs} = 1/K_p + 1/K_{i1} + 1/K_{i2} + \ldots$ etc.).

The y-intercept of equation (1) is $k_1/(k_1 - k_2)$. At infinite inhibitor concentration, $1/[I] = 0$ and $k_1/(k_1 - k_{obs}) = k_1/(k_1 - k_2)$, giving $k_{obs} = k_2$. In other words, at infinite inhibitor concentration, all enzyme is in the form EI and the inactivation rate = $k_2[EI]$. If $k_2 = 0$, (i.e. no dissociation of metal ion occurs from the EI complex) then at $[I] = \infty$, $k_1/(k_1 - k_2) = 1$. If $k_2 > 0$, then the y-intercept will be greater than unity. (Chan, 1983).

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