has about a 30-fold lower affinity for 60S subunits than free 40S subunits. The dissociation rate of 80S ribosomes to 40S and 60S subunits is unaffected by eIF3. This simple thermodynamic cycle gives an association rate for 40S–eIF3 + 60S → 80S–eIF3 of 1.6×10^5 M^{-1} s^{-1}. If one calculates an initial flow through the two paths of association, about 12% of the 40S subunits would associate via the 40S–eIF3 route as opposed to a prior eIF3 dissociation.

This simple scheme neglects conformational changes in the ribosomes and is probably an oversimplification for detailed analysis. Further studies on the effects of other initiation factors with eIF3 should lead to a more detailed mechanism of the initiation of protein synthesis.

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REFERENCES

Behlke, J., Bommer, U. A., Lutsch, G., Henske, A., & Bielka, H. (1986) *Eur. J. Biochem. 157*, 523–530.
Benne, R., & Hershey, J. W. B. (1978) *J. Biol. Chem. 253*, 3078–3087.
Chaires, J. B., Kegeles, G., & Wahba, A. J. (1979) *Biophys. Chem. 9*, 405–412.
Draper, N. R., & Smith, H. (1966) *Applied Regression Analysis*, Wiley, New York.
Fletcher, R., & Powell, M. J. D. (1963) *Comput. J. 6*, 163.
Goss, D. J., & Harrigan, T. (1986) *Biochemistry 25*, 3690–3695.
Goss, D. J., Parkhurst, L. J., & Wahba, A. J. (1980) *Biophys. J. 32*, 283.
Goss, D. J., Parkhurst, L. J., & Wahba, A. J. (1982) *J. Biol. Chem. 257*, 10119.
Goss, D. J., Rounds, D., Harrigan, T., Woodley, C. L., & Wahba, A. J. (1988) *Biochemistry 27*, 1489–1494.
Gualerzi, C., Risuleo, G., & Pon, C. L. (1977) *Biochemistry 16*, 1684–1689.
Lax, S. R., Lauer, S. J., Browning, K. S., & Ravel, J. M. (1986) *Methods Enzymol. 118*, 109–128.
Sperrazza, J. M., & Spremulli, L. L. (1983) *Nucleic Acids Res. 11*, 2655–2679.
Tanford, C. (1967) *Physical Chemistry of Macromolecules*, p 229, Wiley, New York.
Trachsel, H., & Staehelin, R. (19799) *Biochim. Biophys. Acta 565*, 305–314.
Van der Hofstad, G. A. J. M., Buitenhek, A., Bosch, L., & Voorma, H. O. (1978) *Eur. J. Biochem. 89*, 949–953.
Weil, J., & Hershey, J. W. B. (1981) *Biochemistry 20*, 5859–5865.
Weil, J., Hershey, J. W. B., & Levison, S. A. (1978) *FEBS Lett. 87*, 103–106.
Wintermeyer, W., & Gualerzi, C. (1983) *Biochemistry 22*, 690–694.
Wooley, P., & Box, R. (1979) *FEBS Lett. 108*, 433–435.
Zucker, F. H., & Hershey, J. W. B. (1986) *Biochemistry 25*, 3682–3690.

Multiple Sites and Synergism in the Binding of Inhibitors to Microsomal Aminopeptidase†

Maria DiGregorio, Darryl S. Pickering, and William W.-C. Chan*
Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5
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ABSTRACT: The active site of microsomal aminopeptidase has been probed by studying the inhibition of the enzyme in the simultaneous presence of two ligands. The results have been analyzed with the Yonetani–Theorell plot to quantitate the degree of interaction between the two inhibitors. As expected, the enzyme contains a strong binding site for the α-amino group and the hydrophobic side chain of specific substrates. In addition, however, the enzyme can interact with another amine and a second hydrophobic group. Evidence suggests that this extra amine may bind to the zinc in an unprotonated form and that one of the hydrophobic sites is located in the vicinity. Another unexpected finding in this work is a strong synergism between the binding of ammonia and that of zinc ligands such as hydroxamates. This synergism may reflect an induced-fit mechanism that brings the catalytically important zinc atom into the optimal state only in the presence of specific substrates.

The existence of enzymes that specifically cleave the N-terminal residue of peptides has been recognized for some time. [For a review, see Delange and Smith (1971).] These aminopeptidases are widely distributed in nature and may perform a variety of physiological functions.[1] Of particular current interest is the suggestion that an enzyme of this type may participate in the inactivation of neuropeptides (Gros et al., 1985).

In addition to its usefulness as a tool in determining peptide sequence, aminopeptidase has also attracted some attention because of its mechanism (Bryce & Rabin, 1964; Lin & Van Wart, 1982; Makinen et al., 1982; Taylor et al., 1982; Allen et al., 1983). Since the enzyme is known to contain an essential

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[1] In this general introduction, we include for the purpose of background information references to aminopeptidases from many sources, fully recognizing that there are differences in their structural properties and perhaps even in their catalytic mechanism. Subsequent discussions will focus specifically on the microsomal enzyme from pig kidney on which our work is based.

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zinc ion (Himmelhoch, 1969; Wacker et al., 1971), the obvious question is whether there are similarities to other zinc proteases such as carboxypeptidase and thermolysin. In this regard, the study of inhibitors can provide valuable inforamtion concerning the nature of the active site. This approach may also lead to the development of specific inhibitors that can then be used to probe the in vivo role of the enzyme.

Until several years ago, the sensitivity of various aminopeptidases toward inhibitors appeared to follow a pattern very different from that of other zinc proteases. Thus, at that time the most effective inhibitors for aminopeptidases were chloromethyl ketones (Birch et al., 1972), *O-tert*-butylthreonyl peptides (Jost et al., 1972), phenylsulfinyl phenylacetates (Miller & Lacefield, 1979), α-amino aldehydes (Anderson et al., 1982), and the microbial products amastatin and bestatin (Umezawa et al., 1976). These compounds were structurally very different from typical inhibitors of zinc proteases such as thiols, hydroxamates, and phosphoramidates (Cushman et al., 1977; Nishino & Powers, 1978, 1979). However, we found unexpectedly that the microsomal aminopeptidase from porcine kidney was strongly inhibited by α-amino acid hydroxamates (Chan et al., 1982). Similar effects were also reported by other workers on aminopeptidases from different sources (Coletti-Previero et al., 1982; Baker et al., 1983). Subsequently, we demonstrated that thiol derivatives of amino acids were also potent inhibitors of the enzyme (Chan, 1983; Pickering et al., 1985). These results indicated that the catalytic mechanism of aminopeptidase might be analogous to that of other zinc proteases. Nevertheless, the situation is uncertain as recent work on α-aminoboronic acids (Shenvi, 1986) suggests similarities to serine proteases.

Aside from the question of affinity, the most important aspect in the study of an inhibitor concerns its mode of binding. Clearly, the method that provides the most detailed and unambiguous information is X-ray crystallography of the enzyme–inhibitor complex. However, a great deal of insight into the spatial relationship of the various binding regions can be obtained from studying the concurrent effects of two inhibitors on the enzyme. For example, Baker et al. (1983) have shown that the proximity of certain binding sites in *Aeromonas* aminopeptidase can be determined with some precision by this approach. A vital part of such a study is the analysis of the inhibition data with the Yonetani–Theorell plot (Yonetani & Theorell, 1964), which yields a quantitative parameter for the interaction between two inhibitors. In this paper, we have used the same method to probe the active site of microsomal aminopeptidase. Unlike previous workers (Baker et al., 1983), we have paid special attention to the amine binding site, and we have also examined a wider range of ligands. Our results reveal a much higher degree of complexity in the binding of ligands to this enzyme.

Materials and Methods

Source of Materials. Microsomal aminopeptidase (EC 3.4.11.2) of porcine kidney origin was obtained from Sigma Chemical Co. as a suspension in 3.4 M ammonium sulfate. Inhibitors and suppliers were as follows: ethylenediamine, Fisher Chemical Co.; 1,3-diaminopropane, MCB Chemicals; β-alanine, Eastman Organic Chemicals; and D-leucine hydroxamate, Enzyme Systems Products. The synthesis of 1-caprohydroxamate from 1-caproic acid was accomplished by following the procedure of Nishino and Powers (1978). The product was recrystallized from ethyl acetate–hexane mixture, mp 64–65 °C. Other reagents and inhibitors (including all hydroxamates not previously mentioned) were purchased from Sigma Chemical Co.

Kinetic Experiments. For activity measurements, the enzyme was diluted into 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.5) containing 10 μM $CaCl_2$ and 0.2 mg of bovine serum albumin/mL. Stock solutions of inhibitors were adjusted to pH 7.5 before use. Assays were conducted at 25 °C for 5 min in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl and 1 μg/mL aminopeptidase. For the determination of each K_i, assays were performed at four different concentrations of the substrate (L-leucine *p*-nitroanilide) ranging from 0.4 to 2 mM and five inhibitor concentrations from 0 to about $2K_i$. The initial velocities were obtained from the decrease in absorbance at 405 nm and were analyzed by Lineweaver–Burk and Dixon plots.

Each experiment on the concurrent effects of two inhibitors consisted of 20 assays with five concentrations of one inhibitor and four concentrations of the other. The reaction conditions were identical with those described above except that substrate concentration was kept constant at 1 mM. In the equation (for explanation of symbols, see footnote 2) derived by Yonetani and Theorell (1964)

$$\frac{1}{V_i} = \frac{1}{V_m} + \frac{K_m}{sV_m}\left(1 + \frac{i_2}{K_{EI_2}}\right) + \frac{K_m i_1}{sV_m K_{EI_1}}\left(1 + \frac{i_2}{\alpha K_{EI_2}}\right)$$

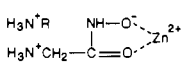
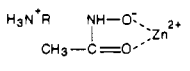
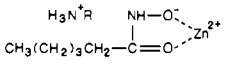
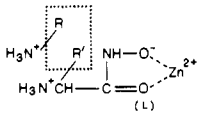
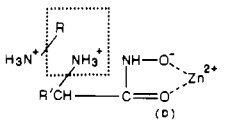
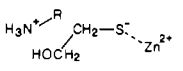
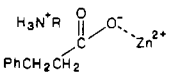
it can be seen that a plot of $1/V_i$ verus i_1 would yield a straight line for each value of i_2. These lines will intersect at $-\alpha K_{EI_1}$ and hence α can be calculated by using the value of K_{EI_1} from the appropriate Dixon plot. In practice, there is some subjectivity in estimating the intersection of four lines. We have therefore chosen to determine the slope of each line from the above plot by linear regression. It can be shown that a secondary replot of these slopes against i_2 yields a straight line with an intercept of $-\alpha K_{EI_2}$. Thus, values of α are obtained by two successive linear regression steps with correlation coefficients generally higher than 0.98 in our experiments.

The Yonetani–Theorell equation is symmetrical with respect to the two inhibitors. Consequently, it is possible to estimate α by plotting $1/V_i$ against either i_1 or i_2. The two values so obtained will not generally be identical since their calculations are based on either K_{EI_1} or K_{EI_2}, the estimates of which are not error free. In most experiments, values of α obtained from the two alternative plots are very close. However, in order to ensure consistency, we have followed a single procedure using the K_i of the same inhibitor wherever α values are to be compared. For example, the α values in Tables I and II were calculated by using the K_i of either ammonia or isoamylamine (rather than the K_i of various zinc ligands).

Since the values of α in this work varied from less than 0.1 to virtually infinity, the concentrations of inhibitors must be carefully selected to obtain good estimates. The optimum range of concentration to use for each inhibitor is from 0 to $2\alpha K_i$, and therefore an approximate value of α must be estimated in a preliminary experiment. For low values of α, the range is sometimes extended to $4\alpha K_i$, while the range is usually kept to about $5K_i$ when α approaches infinity. Under our conditions, α values greater than 10 cannot be estimated with sufficient accuracy and should be considered as indistinguishable from infinity.

[2] Abbreviations: i_1 and i_2, concentrations of the first and second inhibitor, respectively; K_{EI_1} and K_{EI_2}, equilibrium constants for the dissociation of the first and second inhibitor, respectively, from the binary complex; αK_{EI_1} and αK_{EI_2}, equilibrium constants for the dissociation of the first and second inhibitor, respectively, from the ternary complex.

Table I: Concurrent Effects of Ammonia or Isoamylamine and a Zinc Ligand on Aminopeptidase

proposed mode of binding[a]	interaction constant (α) vs ammonia (R = H)	vs isoamylamine (R = $CH_2CH_2CH(CH_3)_2$)	K_i of zinc ligand (mM)
		∞	
	0.14	0.39	3.0
	0.12	0.17	0.81
	0.16	1.0	0.12
	0.09	4.4	0.026
	2.7	7.8	0.055
	0.15	1.4	15
	0.59	0.89	65

[a] Areas of possible steric hindrance are enclosed by dotted lines. R' = $CH_2CH(CH_3)_2$.

Results and Discussion

Binding of the α-Amino Group and the Hydrophobic Side Chain. Our initial goal was to clarify the mode of binding of L-leucine hydroxamate, which we found some time ago to be a strong inhibitor of microsomal aminopeptidase (Chan et al., 1982). By analogy with thermolysin, for which the structure of the hydroxamate inhibitor complex is known (Holmes & Mathews, 1981), we assumed that the hydroxamate moiety would be bound to the active-site zinc ion. In order to probe the binding site of the α-amino group and the hydrophobic side chain, we studied the effects of either ammonia or isoamylamine on the inhibition by various hydroxamates. In these experiments, the concentration of ammonia (or isoamylamine) and that of the hydroxamate inhibitor were both varied, and the interaction constant (α) for each pair of ligands was determined from the Yonetani–Theorell plot. As a control experiment, we tested ammonia and isoamylamine against each other and found the binding to be mutually exclusive ($\alpha = \infty$). These compounds were therefore assigned to the same site in Table I. We then focused attention on the α-amino group by examining glycine hydroxamate. To our surprise, this inhibitor not only did not interfere with the binding of ammonia but instead increased its affinity toward the enzyme by 7-fold ($=1/\alpha$). A similar but smaller effect was shown toward isoamylamine. The synergism was not dependent on the α-amino group since acetohydroxamate also stimulated the binding of ammonia. In this case, the interaction constant for isoamylamine was nearly equal to that for ammonia, suggesting that in the absence of the α-amino group the hydrophobic side chain encountered little or no steric hindrance. It is interesting to note that the K_i of glycine hydroxamate is nearly 4-fold greater than that of acetohydroxamate. This finding is consistent with the above results showing that the α-amino group of this inhibitor is unable to interact with the site for ammonia.

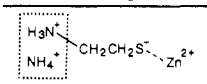
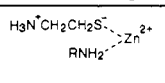
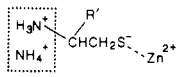
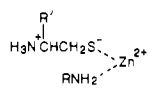
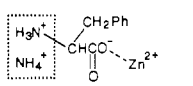
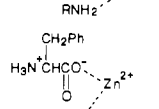
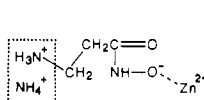
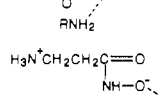
In order to probe the hydrophobic binding site, we tested caprohydroxamate, which was also able to stimulate the binding of ammonia (Table I). However, this compound showed no synergism toward isoamylamine, suggesting that the two hydrophobic side chains interfered with each other to some extent. But the interference was not so strong as to prevent the simultaneous binding of both ligands since the α value remained around unity. The effect of the hydrophobic group of isoamylamine was particularly striking in the case of L-leucine hydroxamate. This inhibitor, which showed a strong synergism with respect to ammonia, was highly antagonistic toward isoamylamine in binding to the enzyme. Thus, although the active site of aminopeptidase had apparently sufficient room for the two hydrophobic side chains, it could not accommodate them well when an α-amino group was also present. These effects were also significantly dependent on the stereochemistry of the hydroxamate, as the D isomer failed to stimulate the binding of ammonia and showed even stronger antagonism toward isoamylamine. A possible explanation of the results in this case might be that in the D configuration the orientation of the α-amino group caused an electrostatic repulsion of the ammonium ion. In this connection, it is interesting that for both cytosolic aminopeptidase and *Aeromonas* aminopeptidase, D-amino acid hydroxamates are stronger inhibitors than the corresponding L isomers (Wilkes & Prescott, 1983). These observations are consistent with our own findings indicating that amino acid hydroxamates are not bound in a substrate-like manner.

Besides providing some information concerning the relative location of the various binding sites, the above results also revealed an interesting and hitherto unknown synergism in this enzyme. In order to explore this phenomenon further, we also tested zinc ligands other than hydroxamates. The binding of ammonia was found to be stimulated to the same degree by β-mercaptoethanol and to a smaller extent by hydrocinnamate (Table I). These effects were reduced or abolished when ammonia was substituted by isoamylamine, presumably because of steric hindrance. However, as in the case of caprohydroxamate, the enzyme seemed able to accommodate simultaneously the hydrophobic side chains of isoamylamine and hydrocinnamate.

Anomalous Binding of Isoamylamine. In contrast to the above results, the binding of ammonia and that of either mercaptoethylamines or α-amino acids were mutually exclusive (Table II). Therefore, unlike α-amino acid hydroxamates, these amino derivatives appear to bind in the same manner as substrates and interact at the amine site. Interestingly enough, β-alanine hydroxamate also interfered strongly with the binding of ammonia. Furthermore, its affinity for the enzyme was about 18-fold higher than that of glycine hydroxamate, suggesting a positive contribution of the β-amino group toward binding.

Since we had found earlier that the binding of ammonia and that of isoamylamine were mutually exclusive, we expected that the effect of isoamylamine would be similar to that of ammonia on these amino derivatives. Surprisingly, however, isoamylamine did not interfere strongly with the binding of the above compounds in spite of its bulkier structure (Table II). These anomalous results, therefore, forced us to postulate a second binding site for isoamylamine. It appears that binding in this alternate mode occurs when the normal amine site is

Table II: Anomalous Interactions of Isoamylamine in the Presence of Some Zinc Ligands[a]

proposed mode of binding[b]	interaction constant (α)	proposed mode of binding[b]	interaction constant (α)
	∞		1.2
	∞		1.8
	∞		3.3
	7.2		0.91

[a] The K_i values of these inhibitors are listed in Table III. [b] Areas of possible steric hindrance are enclosed by dotted lines. R = $CH_2CH_2CH(CH_3)_2$; R′ = $CH_2CH(CH_3)_2$.

Table III: Inhibition Constants of Various Amino Derivatives

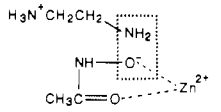
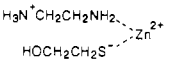
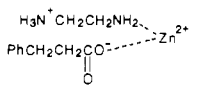
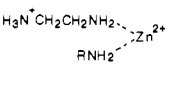
inhibitor[a]	K_i (mM)	pK_a of relevant amino groups[b]
NH_4^+	58	9.3
$H_3N^+CH_2CH_3$	250	
$H_3N^+CH_2CH_2CH(CH_3)_2$	48	10.6
$H_3N^+CH_2CH_2NH_2$	19	7.5 (second group)
$H_3N^+CH_2CH_2CH_2NH_2$	103	8.6 (second group)
$H_3N^+CH_2CH(NH_2)COO^-$ (DL)	0.69	6.7 (2-position) 9.6 (3-position)
$H_3N^+CH_2CH_2COO^-$	47	
$H_3N^+CH_2CH_2S^-$	0.0032	
$H_3N^+CH(R')CH_2S^-$ (L)	0.000051	
$H_3N^+CH(CH_2Ph)COO^-$ (L)	4.9	
$H_3N^+CH_2CH_2CONHO^-$	0.17	

[a] R′ = $CH_2CH(CH_3)_2$. [b] From *Handbook of Biochemistry* (1968).

already occupied and a negatively charged ligand is interacting with the zinc ion. Under these conditions, it is conceivable for the zinc to accept the unprotonated amino group of isoamylamine as a ligand. Although the pK_a of isoamylamine is rather high (Table III), the favorable interaction of the hydrophobic side chain may compensate for the reluctance of the molecule to remain unprotonated. This consideration could explain the difference in the behavior of ammonia and isoamylamine. If this proposed mode of binding for isoamylamine is correct, then β-alanine hydroxamate must be regarded as a monodentate zinc ligand. Quite possibly, the steric constraint imposed by the interaction of the β-amino group at the amine site prevents the hydroxamate moiety from acting as a bidentate ligand in this case.

The Second Amine Site. In order to obtain further information on the location of the alternate amine site, we tested some diamines as inhibitors (Table III). The affinity of ethylenediamine was found to be some 13-fold higher than that of ethylamine, indicating a significant contribution of the second amino group toward binding. The location of this group two carbon atoms away from the other amino function suggests, by analogy with β-mercaptoethylamine and other inhibitors, that it may serve as a zinc ligand. This mode of binding is highly favored by the low pK_a of the second ionization (Table III) and therefore, unlike the case of isoamylamine, may occur without the presence of a hydrophobic side chain or a negative zinc ligand. The positive interaction of the second amino group was relatively specific since 1,3-propanediamine proved to be a poor inhibitor. The weak binding of the γ-amino group could be attributed either to its location or to the much higher pK_a. Further support for the above location of the site was provided by DL-2,3-diaminopropionate, which was bound about 70-fold tighter than β-alanine. (The actual difference in affinity would be double this amount if the inhibition was due to one enantiomer.) Again in this case, the potential of the amino group in the 2-position to act as a zinc ligand would be greatly enhanced by its low pK_a.

Table IV: Concurrent Effects of Ethylenediamine and a Second Inhibitor

proposed mode of binding[a]	α	proposed mode of binding[a]	α
	∞		7.5
	1.4		∞

[a] Areas of possible steric hindrance are enclosed by dotted lines. R = $CH_2CH_2CH(CH_3)_2$.

Binding Mode of Ethylenediamine. In order to confirm that the second amino group of ethylenediamine was bound to the zinc, we tested the effect of this inhibitor in the presence of several compounds that were known from previous results to act as zinc ligands (Table IV). In support of the above hypothesis, the binding of acetohydroxamate and that of ethylenediamine were found to be mutually exclusive. However, when acetohydroxamate was replaced by hydrocinnamate, the interference was minimal. This result indicated that the binding of ethylenediamine was compatible with another monodentate zinc ligand but not with one that is bidentate. As discussed earlier in connection with isoamylamine, a negatively charged ligand such as the carboxylate should facilitate binding of the amine to the zinc. Conversely, therefore, the presence of a poorly ionizable ligand such as the thiol group of β-mercaptoethanol would be expected to weaken the binding of ethylenediamine. In a similar way, the failure of isoamylamine to bind concurrently with ethylenediamine could be attributed to the inability of the zinc ion to accept more than one amino ligand. Thus, all the above results are consistent with the proposed mode of binding for ethylenediamine.

General Discussion. This work has produced some results that were quite unexpected when we originally set out to investigate the binding mode of amino acid hydroxamates. First, we have found evidence of multiple sites for amino groups and for hydrophobic side chains. The potential interactions at these sites may be important in understanding the specificity and catalytic mechanism of the enzyme. Second, and perhaps more significantly, we have observed a synergism in the binding of ammonia and zinc ligands. No similar effect has apparently been reported for any zinc protease. In fact, very few instances of positive interaction between inhibitors seem to have been documented in the literature. Since a free amino group is the primary specificity determinant for aminopeptidase substrates and zinc is a critical catalytic component, an obvious explanation is that the effect constitutes part of an induced-fit mechanism (Koshland, 1960). Such a model would predict that in the absence of specific substrates, the zinc does not exist in a functionally optimal state. Upon binding of a primary amine at the substrate recognition site, a conformational

change would occur that alters the coordination state of the zinc and increases its electrophilic character. Thus, the observed tighter binding of ligands might reflect the enhanced ability of the zinc to polarize the carbonyl bond of specific substrates. Although the mechanism of aminopeptidase has not been elucidated in detail, the sensitivity of this enzyme to inhibitors suggests that it may be similar to that of other zinc proteases. It would, therefore, be of considerable interest to determine whether analogous effects operate in enzymes such as carboxypeptidase and thermolysin.

The detection of a second amine-binding locus in this work has clear implications for the further refinement of inhibitor design. In particular, the relatively high affinity of DL-2,3-diaminopropionate suggests that this compound may provide the basic structure for a new series of inhibitors. Since we have also found two separate sites for hydrophobic side chains, the prospects of obtaining very tight binding inhibitors by the introduction of substituents are certainly promising. Although a number of synthetic inhibitors are already available for aminopeptidase, the most potent of these (i.e., α-amino aldehydes and mercaptoethylamines) are unstable. If derivatives based on 2,3-diaminopropionate could be developed as inhibitors, they would have the considerable advantage of stability.

Our results show that the concurrent effects of different inhibitors can yield valuable insight into the interactions occurring at separate regions of the active site. Unlike X-ray crystallography of enzyme–inhibitor complexes, the above approach provides no direct structural information. However, it is able to determine the strength of interactions and may therefore complement structural and kinetic studies in elucidating enzyme mechanism.

REFERENCES

Allen, M. P., Yamada, A. H., & Carpenter, F. H. (1983) *Biochemistry 22*, 3778–3783.

Baker, J. O., Wilkes, S. H., Bayliss, M. E., & Prescott, J. M., (1983) *Biochemistry 22*, 2098–2103.

Birch, P. L., El-Obeid, H. A., & Akhtar, M. (1972) *Arch. Biochem. Biophys. 148*, 447–451.

Bryce, G. F., & Rabin, B. R. (1964) *Biochem. J. 90*, 513–518.

Chan, W. W.-C. (1983) *Biochem. Biophys. Res. Commun. 116*, 297–302.

Chan, W. W.-C., Dennis, P., Demmer, W., & Brand, K. (1982) *J. Biol. Chem. 257*, 7955–7957.

Coletti-Previero, M.-A., Crastes de Paulet, A., Mattras, H., & Previero, A. (1982) *Biochem. Biophys. Res. Commun. 107*, 465–469.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977) *Biochemistry 16*, 5484–5490.

Delange, R. J., & Smith, E. L. (1971) *Enzymes (3rd Ed.) 3*, 81–118.

Gros, C., Giros, B., & Schwartz, J.-C. (1985) *Biochemistry 24*, 2179–2185.

Handbook of Biochemistry (1968) (Sober, H. A., Ed.) Chemical Rubber Co., Cleveland, OH.

Himmelhock, S. R. (1969) *Arch. Biochem. Biophys. 134*, 513–518.

Holmes, M. A., & Mathews, B. W. (1981) *Biochemistry 20*, 6912–6920.

Jost, R., Masson, A., & Zuber, H. (1972) *FEBS Lett. 23*, 211–214.

Koshland, D. E. (1960) *Adv. Enzymol. Relat. Areas Mol. Biol. 22*, 45–97.

Lin, S. H., & Van Wart, H. E. (1982) *Biochemistry 21*, 5528–5533.

Makinen, K. K., Makinen, P.-L., Wilkes, S. H., Bayliss, M. E., & Prescott, J. M. (1982) *Eur. J. Biochem. 128*, 257–265.

Miller, F., & Lacefield, W. (1979) *Biochem. Pharmacol. 28*, 673–675.

Nishino, N., & Powers, J. C. (1978) *Biochemistry 17*, 2846–2850.

Nishino, N., & Powers, J. C. (1979) *Biochemistry 18*, 4340–4347.

Pickering, D. S., Krishna, M. V., Miller, D. C., & Chan, W. W.-C. (1985) *Arch. Biochem. Biophys. 239*, 368–374.

Shenvi, A. B. (1986) *Biochemistry 25*, 1286–1291.

Taylor, A., Sawan, S., & James, T. L. (1982) *J. Biol. Chem. 257*, 11571–11576.

Umezawa, H., Aoyagi, T., Suda, H., Hamada, M., & Takeuchi, T. (1976) *J. Antibiot. 29*, 97–99.

Wacker, H., Lehky, P., Fischer, E. H., & Stein, E. A. (1971) *Helv. Chim. Acta 54*, 473–485.

Wilkes, S. H., & Prescott, J. M. (1983) *J. Biol. Chem. 258*, 13517–13521.

Yonetani, T., & Theorell, H. (1964) *Arch. Biochem. Biophys. 106*, 243–251.