An Aminopeptidase Occurring in Pig Kidney. II. A Study on the Mechanism of the Hydrolysis*

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ABSTRACT: The hydrolytic activity of the purified aminopeptidase from pig kidney was examined using the amides and *p*-nitroanilides of various amino acids as substrates. Of the naturally occurring amino acids, alanine derivatives were most easily hydrolyzed while more hydrophobic amino acid derivatives were most firmly bound. Simple amino acid amides were hydrolyzed 200 times more slowly than the corresponding amino acid *p*-nitroanilides but were only ten times more weakly bound. The steric influence of the amino acids upon K_M and V_{max} of the aminopeptidase was estimated from experiments utilizing aliphatic acids, amines, and amides, and amino acids and their amides as inhibitors. The formation of a ternary aminopeptidase–substrate–inhibitor complex was most easily seen in the case of the carboxylic acids. There was an alternate increase and decrease in the inhibitory capacity as the length of the hydrocarbon side chain was increased, closely paralleling the well-known stepwise variation in the melting points of a homologous series of hydrocarbon derivatives.

This indicated a highly ordered state at the hydrolytic center of the enzyme. In alcoholic solutions the structure of the aminopeptidase was so changed that hydrophobic and hydrophilic substrates were hydrolyzed at the same rate. Dioxane, dimethyl sulfoxide, and dimethylformamide also gave rise to an uncompetitive type of inhibition. From these results it was possible to recognize three binding sites on the enzyme, one for the N-terminal aliphatic side chain, one for the amino residue of the N-terminal amino acid, and one for the aliphatic side chain of the penultimate amino acid.

In a recent paper we described the preparation of pig kidney aminopeptidase and some of its biochemical properties (Wachsmuth *et al.*, 1966). The enzyme is quite different from the aminopeptidases previously known (Smith and Hill, 1960; Wachsmuth, 1966), and it seemed interesting to examine its specificity against different amino acid derivatives. It is hoped that a study of the activation of these derivatives and of their affinities for the enzyme will shed light on the mechanism of the hydrolysis.

Experimental Procedures

Materials. The pig kidney aminopeptidase used in this work hydrolyzed 31–37 μmoles of leucine *p*-nitroanilide/1 mg of protein per min at 37° when saturated with substrate. The preparation of the enzyme has already been described (Wachsmuth *et al.*, 1966). The amino acid *p*-nitroanilides (kindly synthesized by C. F. Boehringer, Mannheim) and the amino acid amides (mainly synthesized by Dr. H. Determann, Organisch chemisches Institut der Universität, Frankfurt/M) were used as substrates.

Methods. All hydrolyses were carried out in a thermostatically controlled cuvet at 37°. In the preceding paper we described the methods of measuring the hydrolysis rates of the *p*-nitroanilides and of the amides (Wachsmuth *et al.*, 1966). The amount of substrate hydrolyzed was not more than 1.0% of that present in the reaction mixture, and within this range the rate of hydrolysis remained constant during the period of the test.

The enzyme dissociation constants were derived according to Lineweaver and Burk (1934). Inhibition constants for amino acids and their amides were determined by comparing the K_M values with and without inhibitor. Where possible, the concentration of the inhibitor was so adjusted that the Michaelis constant was nearly doubled, and then the concentration of inhibitor necessary to exactly double K_M was calculated; it is reported here as K_I. In the tables the quotient K_I/K_M is given as a measure of the inhibition. Unless otherwise mentioned K_M is the affinity for leucine *p*-nitroanilide hydrolysis.

The influence of alcohols and organic solvents on the dissociation constants of aminopeptidase was tested without preincubation. The hydrolysis was initiated by addition of the enzyme. In every case in order to demonstrate that no denaturation of the enzyme occurred during the measurement period a sample of enzyme was incubated in the appropriate solvent and then tested in an inhibitor-free milieu.

The influence of aliphatic amines, acids, and amides on the aminopeptidase was investigated by measuring V_{max} and K_M for the leucine *p*-nitroanilide hydrolysis at a given concentration of inhibitor substance. They are indicated by K_M' and V_{max}'.

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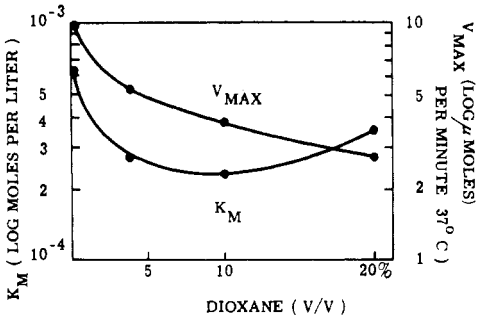

FIGURE 1: Michaelis constants (K_M) and maximal rates of hydrolysis (V_{max}) of alanine *p*-nitroanilide by aminopeptidase tested in 0.06 M phosphate buffer, pH 7.0, and in different concentrations of dioxane (v/v) at 37°. Both ordinate scales are logarithmic.

Results

Determination of the Michaelis Constant (K_M) and the Maximal Rate of Hydrolysis (V_{max}). Table I shows the Michaelis constants (K_M) and Table II the maximal rates of hydrolysis (V_{max}) for the amino acid amides and *p*-nitroanilides. When the amino acid derivatives were arranged according to either the V_{max} values or to the K_M values the order was essentially the same. The much higher V_{max} values of the *p*-nitroanilides are understandable since amino acid *p*-nitroanilides are much more readily hydrolyzed by acid than are the amides. In general the enzyme has a ten times higher affinity for the *p*-nitroanilides than for the corresponding amides. This indicates that aminopeptidase recognizes and thus interacts with the carboxyl substituent of the N-terminal amino acid.

The affinity of aminopeptidase for its substrate increases with the hydrophobic character of the side chain of the amino acid, although for the homologous series alanine, α-aminobutyric acid, norvaline, and norleucine it is nearly the same. In contrast the V_{max} for alanine derivatives is high while the V_{max} of the other members of the series is lower and of the same magnitude. The amides of amino acids containing a secondary amino group were very weakly bound and hardly hydrolyzed at all.

Influence of Organic Solvents on K_M and V_{max}. Since the hydrophobic character of a substrate appears to be important the kinetic properties of aminopeptidase were measured in solvents of low dielectric constant (Tables I, II, and III). The effect of 20% dioxane on the binding affinity of aminopeptidase for amino acid amides is shown in Table I. It is possible to recognize three types of amino acid. For those containing a cyclic side chain the affinity was unchanged. The K_M values of the aliphatic amino acid amides decreased. The K_M value of glycine amide was unchanged. Since glycine *p*-nitroanilide is more weakly bound in dioxane it appears that the introduction of the *p*-nitrophenyl residue leads to a relatively weaker binding in dioxane. This effect is, however, often swamped by the increased binding due to the influence of dioxane on the aliphatic side chain.

TABLE I: The Michaelis Constants (mmolar) for the Aminopeptidase Hydrolysis of Various Amino Acid Derivatives.[a]

	p-Nitroanilide		Amide	
L-Amino Acid	Buffer	20% Dioxane	Buffer	20% Dioxane
Sarcosine			280	
Proline			200	200
Serine			67	35
Glycine	1.75	8.0	25	25
Histidine			25	25
Valine			19.9	8.6
Phenylalanine	3.2	2.0	10.0	10.0
Alanine	0.6	0.37	9.0	1.4
α-Aminobutyric acid			8.8	3.13
Norleucine			7.7	2.5
Norvaline			6.0	
Leucine	0.24	0.23	2.35	1.82

[a] Determined in 0.06 M phosphate buffer, pH 7, at 37°, and in 20% (v/v) dioxane solution.

TABLE II: The Maximum Rates of Hydrolysis of Various Amino Acid Derivatives by Aminopeptidase.[a]

	p-Nitroanilide		Amide	
L-Amino Acid	Buffer	20% Dioxane	Buffer	20% Dioxane
Alanine	100	84	1.15	0.32
Norvaline			0.44	
Norleucine			0.35	0.12
Leucine	71	15	0.32	0.13
Phenylalanine	83	14	0.29	0.085
Serine			0.2	0.2
Valine			0.11	0.1
Glycine	22	42	0.085	0.21
α-Aminobutyric acid			0.6	0.21
Histidine			0.058	0.032
Proline			0.0042	0.0042
Sarcosine			0.0002	

[a] Values are per cent of V_{max} of alanine *p*-nitroanilide measured in 0.06 M phosphate buffer at 37° and in 20% (v/v) dioxane solution, pH 7.

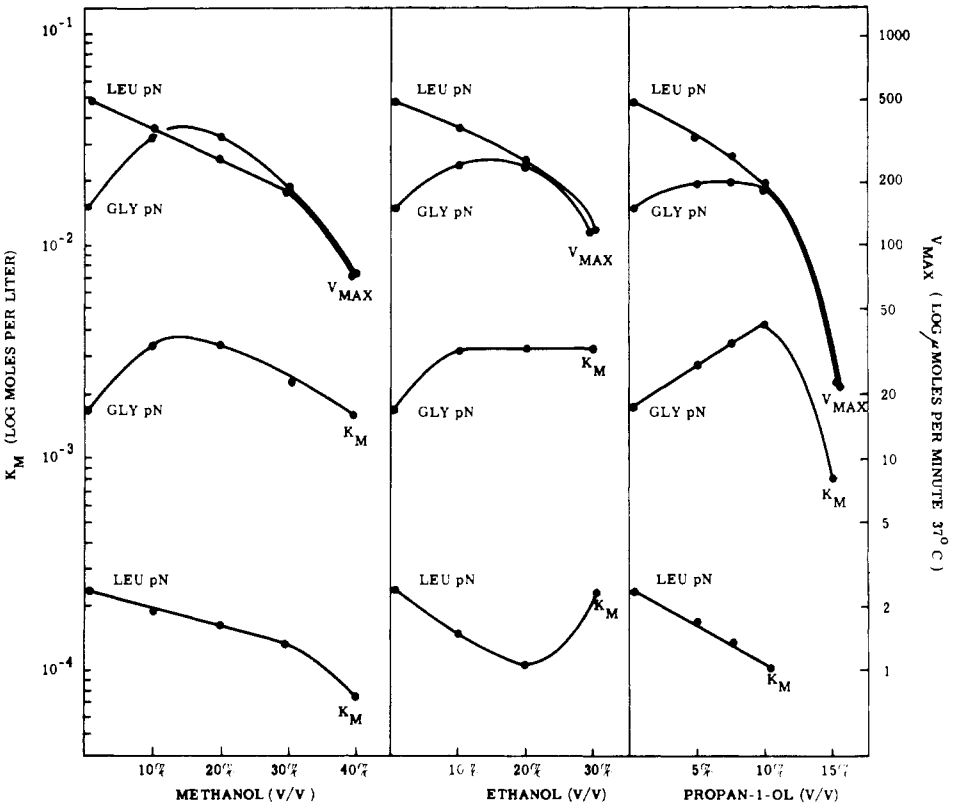

FIGURE 2: K_M and V_{max} values for hydrolysis of glycine and leucine *p*-nitroanilide by equal amounts of amino peptidase at different concentrations of methanol, ethanol, and 1-propanol (v/v) tested in 0.06 M phosphate buffer, pH 7.0, at 37°. Both ordinate scales are logarithmic.

TABLE III: Influence of Organic Solvents on Hydrolyses by Aminopeptidase.[a]

Substrate (*p*-nitroanilide)	Solvent		
	Buffer	Dimethyl Sulfoxide	Dimethylformamide
K_M Leucine	0.24	0.28	0.28
Alanine	0.6	0.09	0.51
Glycine	1.75	7.9	4.0
V_{max} Leucine	72	25	19
Alanine	100	56	36
Glycine	22	56	36

[a] The Michaelis constants (mmolar) and the V_{max} values (per cent of alanine *p*-nitroanilide hydrolysis) were determined in 0.06 M phosphate buffer, pH 7.0, at 37°, and in 10% (v/v) solvent.

The hydrolysis rates of glycine amide and glycine *p*-nitroanilide were doubled in dioxane (Table II) while those of the other amino acid derivatives, especially the hydrophobic ones, were decreased. Thus there was smaller spread of V_{max} values in dioxane although alanine derivatives remained the best substrates. This converging of the V_{max} values was found in all the other solvents tested (Table III).

The results from the enzymic hydrolysis in the presence of organic solvents emphasize the importance of the steric configuration and hydrophobic character of the N-terminal amino acid. The hydrolysis constants of the glycine derivatives show that in the absence of an α-C substituent these derivatives are not in the optimal configuration for hydrolysis.

As the concentration of the organic solvent increases, the substrates are more strongly but less specifically bound at the active center, presumably due to a configurational change of the enzyme. As increases in dioxane concentration did not lead to a continual decrease of K_M for alanine *p*-nitroanilide (Figure 1), it is improbable that these effects could be due simply to a change in the dielectric constant of the medium at the active center.

Influence of Various Alcohols on K_M and V_{max}. Smith has already described leucine aminopeptidase inhibition in the presence of alcohol, but only at one substrate concentration (Hill and Smith, 1957). We have been able to show that the longer the aliphatic side chain, the more effective is the alcohol as a denaturant (Wachsmuth *et al.*, 1966). In order to examine the hydrophobic interactions between enzyme and substrate, the K_M and

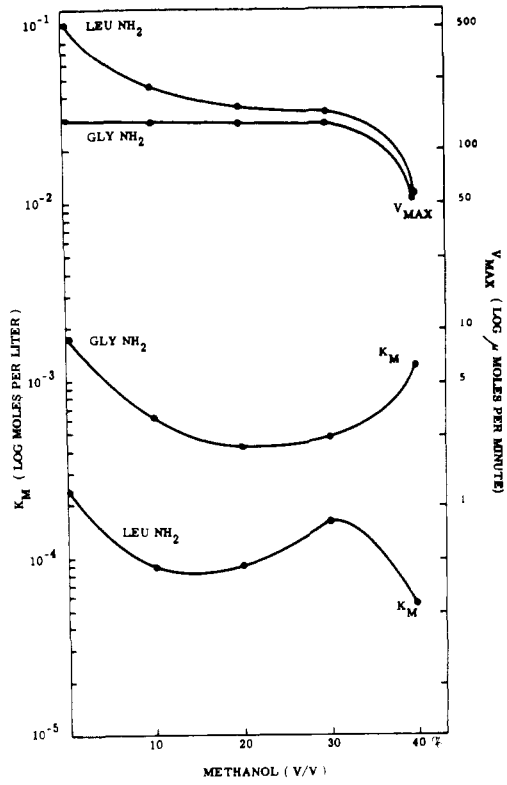

FIGURE 3: K_M and V_{max} values for hydrolysis of glycine and leucine amide at different concentrations of methanol. Experimental conditions were the same as in Figure 2. Both ordinate scales are logarithmic.

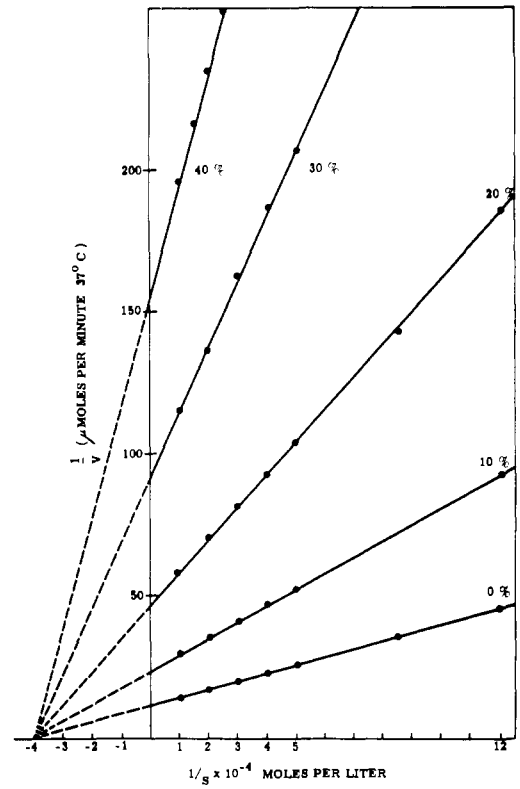

FIGURE 4: The typical noncompetitive hydrolysis inhibition of leucine *p*-nitroanilide tested in 0.06 M phosphate buffer, pH 7.0, and Methyl Cellosolve (v/v) at 37°. Values are plotted after the method of Lineweaver and Burk (1934). The values shown next to each curve are the volume per cent Methyl Cellosolve in which the measurements were made.

V_{max} values of the enzyme were measured in different aliphatic alcohols at varying concentrations.

Leucine *p*-nitroanilide was chosen as an example of a hydrophobic substrate and glycine *p*-nitroanilide as an example of a hydrophilic substrate. The inhibition induced by the alcohols was uncompetitive.

Figure 2 shows that the V_{max} values of leucine *p*-nitroanilide decreased as the concentration of solvent was increased. The hydrolysis of glycine *p*-nitroanilide was at first activated by the presence of alcohols, as was also found for the other organic solvents. Since the hydrolysis of glycine amide was not activated by methanol (Figure 3) it appears that the activation is due to the *p*-nitrophenyl residue. At higher concentrations of alcohols the hydrolysis rates of the two substrates are the same and decrease together.

The effect of alcohols on the K_M values are difficult to interpret, but Figure 3 shows that the effects on both the hydrophilic and hydrophobic amides were the same. The difference between the K_M values of glycine *p*-nitroanilide and glycine amide in methanol indicates that the *p*-nitrophenyl residue weakens the interaction between enzyme and substrate in solvents less polar than water. Thus the major effect of the alcohols is to decrease the V_{max} values for the hydrophobic leucine derivatives, perhaps indicating a change in configuration of the enzyme tending to pull the bound substrate away from the hydrolytic site.

Methyl Cellosolve was chosen as an example of a solvent less polar than the alcohols. The Lineweaver and Burk plots (Figure 4) show that the inhibition of leucine *p*-nitroanilide hydrolysis was strictly noncompetitive whereas the glycine *p*-nitroanilide hydrolysis was activated. These results were a further indication of the postulated conformational change of the enzyme in the presence of the alcohols.

Inhibition by Amino Acids and Amino Acid Amides. The amino acids inhibited competitively when present in such concentrations that K_M was doubled. At higher concentrations of inhibitor it became apparent that the inhibition is mixed competitive and uncompetitive, with the former type dominating. Table IV shows that the more hydrophobic amino acids are the most effective inhibitors. Glycine inhibits 500 times less than norleucine. D-Leucine was 30 times less strongly bound than L-leucine and D-alanine 12 times less than L-alanine. Although the D-amino acids were thus bound by the enzyme, no D-amino acid peptides were hydrolyzed. Comparing norleucine, leucine, and isoleucine it

TABLE IV: Inhibition of the Aminopeptidase Hydrolysis of Leucine *p*-Nitroanilide Hydrolysis by Amino Acids and Amino Acid Amides.[a]

	Amino Acid (K_I/K_M)	Amino Acid Amide (K_I/K_M)
L-Norleucine	5.0	10.0
L-Phenylalanine	7.0	25
L-Leucine	12.3	35
L-Isoleucine	14	
L-α-Aminobutyric acid	15	
L-Valine	30	93
L-Arginine	36	31
L-Glutamine	40	
L-Histidine	46	80
L-Alanine	63	70
L-Lysine	90	
L-Glutamic acid	230	
L-Serine	230	230
D-Leucine	360	
L-Aspartic acid	390	
D-Alanine	770	
Glycine	1000	300
L-Proline	1800	140
L-Asparagine	3100	
Sarcosine	3400	192

[a] Values are the concentration of inhibitor required for 50% inhibition divided by K_M for leucine *p*-nitroanilide. Conditions as in Table I.

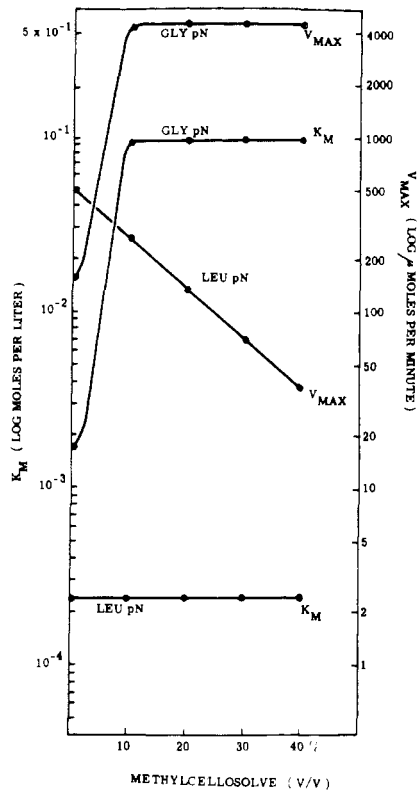

FIGURE 5: K_M and V_{max} values for hydrolysis of glycine and leucine *p*-nitroanilide at different concentrations of Methyl Cellosolve. Experimental conditions were the same as in Figure 2.

is apparent that the branching of the aliphatic side chain of the amino acid results in a decreased affinity for the enzyme. Amino acids containing a secondary amino group were very weakly bound, as can be seen from the values for proline and sarcosine. Glutamine inhibited the enzymic activity of aminopeptidase ten times more than did glutamic acid, but asparagine ten times less than aspartic acid. Therefore an amide group in the β position decreases the affinity, whereas one in the γ position increases it. The amides of the hydrophobic amino acids were more weakly bound than the free amino acids and the amides of the hydrophilic ones more strongly bound. Thus the inhibition constants of the amides showed less spread than those of the free amino acids.

Inhibition by Aliphatic Acids, Amines, and Amides. The inhibition of the hydrolysis of leucine *p*-nitroanilide by aliphatic acids, amines, and amides was investigated in order to obtain information about the functions of the different chemical groups in the substrate.

With leucine *p*-nitroanilide as substrate, Figure 6 shows that the competitive inhibition by the aliphatic amines increased linearly with length of the amine side chain.

The straight chain fatty acids also inhibited the hydrolysis, but this time uncompetitively. The inhibition did not increase linearly with the length of the side chain, and the acids were some eight times more weakly bound than the corresponding amine. From propionamide onwards, the amides cause a decrease in the hydrolysis rate due to the stronger binding of the substrate, leucine *p*-nitroanilide.

Although the inhibition constants (K_M' and V_{max}') are dependent on concentration, it appears from Figure 6 that the logarithm of the K_M' of an amino acid is additively composed of log $K_{M'amine}$ and log $K_{M'acid}$ and furthermore that log $K_{M'aminoacidamide}$ is composed of log $K_{M'amine}$ and log $K_{M'amide}$. Since the K_M' values of the aliphatic acid amides decrease after propane the amino acid amide curve crosses under the amino acid curve. It thus follows that the greatest contribution to the binding of the substrate by the aminopeptidase is made by the amino group of the substrate.

These last-mentioned inhibition experiments utilized the inhibition of the hydrolysis of leucine *p*-nitroanilide. With glycine *p*-nitroanilide, however, the complicating effect of interaction between enzyme and the lipophilic substituent at the α-carbon atom is absent. Thus the results should directly reflect influences on the catalytic center. This influence is seen as an alternating

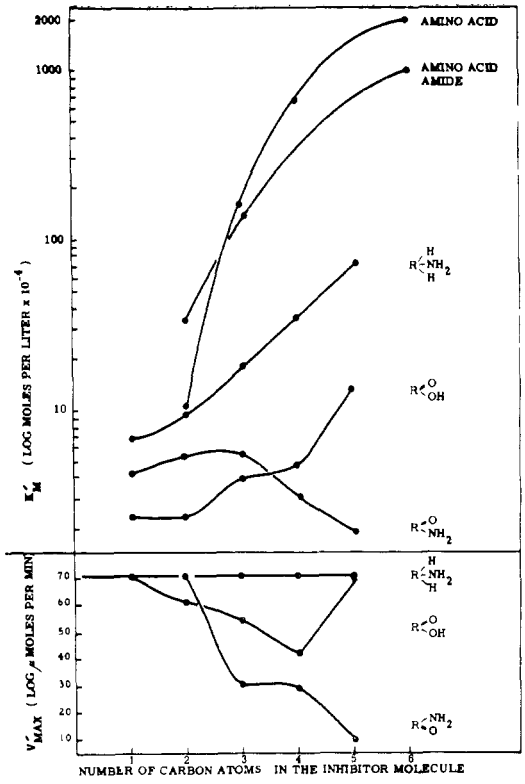

FIGURE 6: K_M and V_{max} for the enzymic hydrolysis of leucine *p*-nitroanilide were measured in 0.06 M phosphate buffer, pH 7.0, at 37° ($K_M = 2.4 \times 10^{-4}$) and in the presence of various aliphatic acids, amides, amines, amino acids, and amino acid amides. The concentration of inhibitor was in each case 0.5 M. The ordinate scale for K_M' is logarithmic, for V_{max} (per cent of alanine *p*-nitroanilide hydrolysis) linear.

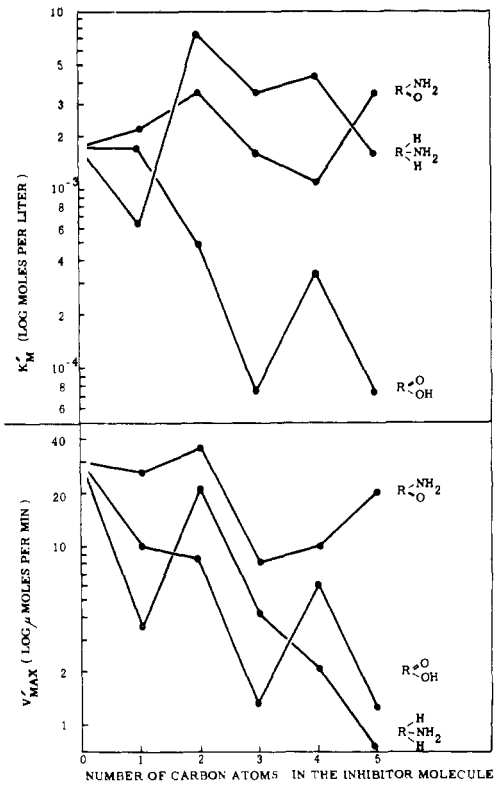

FIGURE 7: K_M and V_{max} for the enzymic hydrolysis of glycine *p*-nitroanilide were measured in 0.06 M phosphate buffer, pH 7.0, at 37° ($K_M = 1.75 \times 10^{-3}$ M) and in the presence of various aliphatic acids, amides, and amines. The concentration of inhibitor was in each case 0.5 M. The ordinate scale for K_M' and V_{max}' (per cent of alanine *p*-nitroanilide hydrolysis) is logarithmic.

increase and decrease in the degree of inhibition with increasing chain length of inhibitor (Figure 7). The inhibition constants, K_M' and V_{max}', for even-numbered aliphatic side chains were relatively higher than those for odd-numbered ones. This is best seen in the values for aliphatic acids where the similarity to the steplike variation in the melting points of the aliphatic acids and their derivatives is very striking. In the presence of the fatty acids, the Lineweaver and Burk curve was moved upwards parallel to the original. This indicates that the inhibitor is only bound by the enzyme–substrate complex. A possible explanation would be that the carboxyl group of the inhibitor is bound by the amino group of the substrate to yield directly a ternary complex.

The alternating interaction between aminopeptidase and inhibitor is recognizable for the inhibition of leucine *p*-nitroanilide hydrolysis by the aliphatic amides and acids. The interactions between the enzyme and the leucine side chain do however cause a considerable smearing.

Inhibition by α-, β-, γ-, and ε-Amino Acids. To test the effect of changing the distance between the amino and carboxyl groups of an amino acid, the inhibition by α-, β-, γ-, and ε-amino acids and their amides was investigated (Figure 8). Even with large amounts of enzyme the derivatives of the β-, γ-, and ε-amino acids were not themselves hydrolyzed, but they were good inhibitors of the enzymic hydrolysis of leucine *p*-nitroanilide. As the distance between the amino and carboxyl groups increased, so also did the degree of inhibition, although the curve flattened off with γ-aminobutyric acid. For the amino acid amides, as the distance between the amino and carboxamide groups was increased the inhibitory influence initially decreased, but then increased sharply again with γ-butyric acid amide. The relatively slight inhibition produced by β-alanine amide when compared to β-alanine is analogous to the relationship between asparagine and aspartic acid (Table IV). The much higher inhibition of γ-butyric acid amide compared with the free amino acid corresponds to the relationship between glutamine and glutamic acid. Thus an amido group β to the amino group reduces the inhibition whereas a γ-amido group increases it.

Inhibition by Various N-Substituted Amino Acids. It has already been noted that proline and sarcosine are

TABLE V: N-Substituted Glycines as Inhibitors for the Enzymic Hydrolysis of Leucine *p*-nitroanilide.[a]

Inhibitor	V_{max}'	K_M'
Buffer	100	0.24
Glycine	100	3.0
N-Monomethylglycine	50	0.6
N-Dimethylglycine	100	6.8
N-Trimethylglycine	77	0.38
N-Acetylglycine	50	2.16
N-Carbobenzoxyglycine	85	0.43

[a] The Michaelis constants (K' = mmolar) and V_{max}' (per cent of leucine *p*-nitroanilide hydrolysis) were determined for leucine *p*-nitroanilide in 0.06 M phosphate buffer, pH 7.0, at 37°, and in 1.5 M inhibitor.

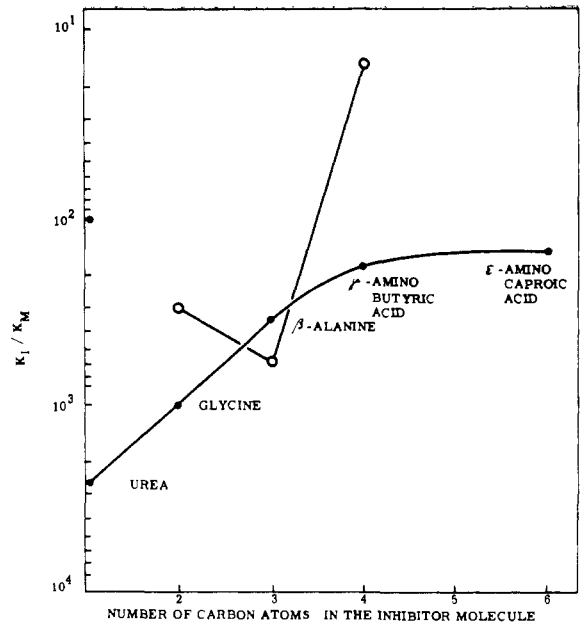

FIGURE 8: Inhibition of leucine *p*-nitroanilide hydrolysis by α-, β-, γ-, and ε-amino acids (●——●) and amino acid amides (○——○), tested in 0.06 M phosphate buffer, pH 7.0, at 37°. Values are the concentrations of inhibitor required for 50% inhibition divided by K_M for leucine *p*-nitroanilide (K_I/K_M). K_I/K_M for NH_4Cl = 10^2. The ordinate scale is logarithmic.

bound by aminopeptidase, even though much less than glycine, and that their amides are hydrolyzed. Table V shows the K_M' and V_{max}' values of differently substituted glycines for the inhibition of the hydrolysis of leucine *p*-nitroanilide. The alternating strong and weak inhibition by the various methylglycines strongly resembles the alternating inhibition of the glycine *p*-nitroanilide hydrolysis by the aliphatic acids. Glycine is a good inhibitor, monomethylglycine poor, dimethylglycine good again, and trimethylglycine poor.

Discussion

A comparison between the hydrolysis rates and binding affinities of aminopeptidase of pig kidney for amino acid amides and *p*-nitroanilides did not reveal any principal differences between the two types of substrate.

The relation of the amino acid derivatives to one another in both groups was the same, although the two groups differed in the numerical values of their constants. Of all the amino acids, alanine derivatives were most easily hydrolyzed and derivatives of hydrophobic amino acids were most strongly bound. The inhibition experiments with organic solvents and with the free amino acids, aliphatic acids, amides, and amines indicate a hydrophobic binding site on the protein for lipophilic substituents at the α-carbon atom of the substrate.

For the hydrolysis of the hydrophobic leucine *p*-nitroanilide the best inhibitors were those containing a hydrophobic side chain. Thus the hydrophobic character is primarily responsible for determining the extent of the inhibitory influence. Substrates derived from *n*-butane or larger *n*-hydrocarbons (such as α-aminobutyric acid, norvaline, and norleucine) are optimally bound while only the derivatives of *n*-propane are optimally hydrolyzed (three unbranched carbon atoms such as in alanine).

It appears that although the butane backbone directs the substrate optimally onto the catalytic center during the hydrolysis there is a greater steric hindrance to the conformational change of the enzyme with butane-type substrates than with propane-derived substrates. With the ethane derivatives such as glycine this steric directing factor is absent.

It is significant that a rhythmic increase and decrease of V_{max}' and K_M' occurs with homologous series of aliphatic acids and their derivatives. The even-numbered acids and amines cause a relative increase while the odd numbered cause a relative decrease. Since these variations closely parallel the melting points of the aliphatic acids, amines, and amides it is concluded that a highly organized structure exists at the hydrophobic binding site of the enzyme which to some extent corresponds to the crystalline structure of the substrate.

A hydrophobic binding site was also demonstrated by kinetic experiments with a homologous series of alcohols, whose effect above a certain concentration was to cause the hydrolysis rates of all the amino acid derivatives to converge on one another. Methyl Cellosolve produces the same effect, but due to its weaker denaturing properties *p*-nitroanilides of leucine and glycine have unchanged Michaelis constants over a wide range of concentrations of this organic solvent.

Hydrolyses in the presence of organic solvents and alcohols indicate, in addition to changing the dielectric constant of the medium, that an easily reversible conformational change in the enzyme structure occurs. It

could be imagined that this change tends to pull the substrate-binding site, and thus the bound substrate, away from the hydrolytic center. This effect is absent for the glycine derivatives which lack a hydrophobic substituent at their α-carbon atom.

Inhibition experiments with a series of aliphatic homologs showed that a primary amino group is preferred and better bound by the aminopeptidase than a carboxyl or an amide group. Thus amines produce competitive inhibition, whereas a free carboxyl group lowers the binding affinity considerably. The amino acid has to have the L configuration and needs a primary amino group for the optimal hydrolysis and binding of its derivatives. The primary amino group has to be in the α position to the carboxyl group since in the β position no hydrolysis occurs. The strong affinity of the α-amino group for the enzyme is significantly reduced by an amide nitrogen in the β position (asparagine and β-alanine amide). Inhibition of aminopeptidase by amino acids is therefore a mixed form of competitive and uncompetitive inhibition but with the former type dominating.

The Michaelis constant of aminopeptidase which is ten times smaller for amino acid *p*-nitroanilides than for the corresponding amides demonstrates that there should be also a binding site for the penultimate amino acid. The binding site for the penultimate amino acid can influence the hydrophobic binding site, as can be seen from inhibition constants of the carboxylic acids and α-amino acids compared to the corresponding amides.

Thus aminopeptidase must contain at least two binding sites for the N-terminal amino acid of peptides (one for the primary amino group, a second for the hydrophobic residue at the α-carbon atom), and a third for the penultimate amino acid in a peptide and its derivatives.

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Biosynthesis of Uridine Diphosphate D-Xylose. II. Uridine Diphosphate D-Glucuronate Carboxy-lyase of *Cryptococcus laurentii**

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ABSTRACT: Uridine diphosphate D-glucuronate carboxy-lyase of *Cryptococcus laurentii* has been partially purified and its properties have been investigated. Uridine diphosphate D-xylose and carbon dioxide are the only products of enzyme action. The pH optimum is between 7.0 and 7.5.

K_m for uridine diphosphate D-glucuronic acid is 1.1 $\times$ 10^{-3} M; the enzyme has an absolute and specific requirement for nicotinamide–adenine dinucleotide (NAD); K_a = 3 $\times$ 10^{-6} M. Reduced nicotinamide–adenine dinucleotide inhibits competitively with NAD; K_i = 2 $\times$ 10^{-6} M. The enzyme is inhibited by *p*-mercuribenzoate; the inhibition is reversed by cysteine. Label is retained when uridine diphosphate D-glucuronate-3-*t* or -4-*t* is decarboxylated by the enzyme.

A number of species of the genus Cryptococcus produce extracellular polysaccharides which contain D-xylosyl moieties (Benham, 1956); that secreted by *Cryptococcus laurentii* (NRRL Y-1401) consists of D-mannose, D-xylose, and D-glucuronic acid in the approximate molar ratio 5:2:1 (Abercrombie *et al.*, 1960; see also Jeanes *et al.*, 1964). In higher plants it has been shown that D-glucuronosyl and D-xylosyl moieties

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