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The Assay and Reaction Kinetics of Leucine Aminopeptidase from Swine Kidney

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Kinetic investigation of leucine aminopeptidase (EC 3.4.1.1) has been hampered by lack of suitable methods for following the hydrolytic reaction. The method of Grassmann & Heyde (1929) has been widely used, but its lack of precision makes it suitable only for investigating the specificity of the enzyme. For kinetic investigations, it is desirable to have a continuous record of the reaction and a pH-stat assay has been developed for this purpose.

The enzyme is inhibited by alcohols (Hill & Smith, 1957), and it has been suggested that this is due to competition of the alcohol for the site binding the substrate side chain. A low rate of esterase activity has also been reported (Smith & Polglase, 1949; Smith & Spackman, 1955; Shippey & Binkley, 1958) with leucine esters of small aliphatic alcohols as substrates. Owing to the key importance of the esterase activity and inhibition by alcohols for any theory of the mode of action of the enzyme, we have investigated these problems in some detail.

MATERIALS AND METHODS

Water. Glass-distilled water was passed successively through columns of De-Acidite FF, Zeo-Karb 225 and 215 (mixed) and Bio-Deminrolit (The Permutit Co. Ltd., London).

Sodium chloride. This was either Specpure (Johnson Matthey and Co. Ltd., Hatton Garden, London) or recrystallized from EDTA solution.

Hydrochloric acid. AnalaR hydrochloric acid was diluted and distilled. Solutions of the required dilution were standardized potentiometrically against recrystallized tris (Fossum, Markunas & Riddick, 1951).

Sodium hydroxide. AnalaR sodium hydroxide solution (5N) was passed through a column of Zeo-Karb 225 previously equilibrated with AnalaR sodium chloride.

Substrates. L-Leucine amide, L-leucylvaline, L-leucyl alanine, and L-leucine benzyl ester were obtained from the Yeda Research and Development Ltd. (Rehovoth, Israel). L-Leucylglycine was obtained from Roche Products Ltd. (Welwyn Garden City, Herts.). All the compounds were homogeneous by paper chromatography in butanol-acetic acid-water (4:1:5, by vol.).

Glycerol and butanol. The commercial products were redistilled.

N-Ethylmorpholine. This was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and twice redistilled. It was converted into the hydrochloride by passing anhydrous HCl through a solution in ethanol. The solid was recrystallized twice from acetone-ethanol (2:1, v/v).

Enzyme. This was prepared according to the published method (Hill, Spackmann, Brown & Smith, 1958) as far as the acetone precipitate. Chromatography (Visvanatha, 1959) was substituted for starch-column electrophoresis. A linear gradient of sodium chloride in 5 mm-N-ethylmorpholine buffer, pH 8.0 or 8.4, made 5 mm with respect to magnesium chloride, was employed. The enzyme emerged as a narrow band at the front of the main protein peak at about 0.1 M-sodium chloride. A typical chromatogram is shown in Fig. 1. The contents of the tubes containing the enzyme were combined and rechromatographed under identical conditions, when the protein and activity emerged as a single peak.

The specific activity is expressed as μ moles of L-leucine amide hydrolysed/min./mg. of protein at pH 8.4, I 0.1, 40° and substrate concentration 2.5 mM. For comparison purposes, these units can be converted to 'zero-order proteolytic coefficients' (Smith & Spackman, 1955) by multiplication by 14.

Fractions of specific activity 450–600 were used. The Mn^{2+} ion-activated enzyme was obtained by incubation with 2 mM-manganese chloride for sufficient time to give maximum activity (usually 10 min.).

Protein. This was measured from the extinction at 280 $m\mu$ by assuming that 1 mg. of protein/ml. has an extinction of 0.84 (Spackman, Smith & Brown, 1955).

pH-stat measurements. The technique was essentially that described by Herries, Mathias & Rabin (1962). Magnetic stirring was employed and washed nitrogen gas was blown over the surface. The substrate was taken to the required pH with sodium hydroxide (1 N) delivered through a micrometer-syringe burette. The addition of alkali could be carefully controlled and the volume was negligible. The ionic strength was adjusted with sodium chloride. The reaction was started by addition of enzyme previously adjusted to the same pH. The final volume was in all cases 3 ml.

The pH was maintained constant by the addition of sodium hydroxide for ester hydrolysis, or of hydrochloric acid for peptide or amide hydrolysis. Initial velocities were obtained by measuring the slope of the tangent at the origin of the progress curves. The progress curves were followed for 1–3 min. and were unaffected by preincubating the substrate and activating metal ion before the addition of the enzyme.

PRINCIPLE OF THE METHOD

The method depends on the differences in pK values of amino acids and dipeptides. Thus the pK of the amino group of leucylglycine is 8.25 and on hydrolysis this yields two amino acids with pK values of 9.75 and 9.78. Allowing for the ionization of the new carboxyl group produced, alkali will be generated in the hydrolytic reaction at pH values between the pK of the substrate and the lower of the amino pK values of the products. At any given pH the amount of acid needed for the complete reaction can be calculated from the pK values. If $\bar{h}$ (L) is the average number of ionizable protons bound/mole of L (to both carboxyl and amino groups), then for the hydrolysis of L-leucylglycine:

$$\Delta\bar{h} = \bar{h}(\text{leucine}) + \bar{h}(\text{glycine}) - \bar{h}(\text{leucylglycine})$$

where $\Delta\bar{h}$ is the number of protons required to keep the pH constant for complete hydrolysis of 1 mole of substrate. A plot of this function at I 0.1 is shown in Fig. 2. The best pH range is obviously 8.4–8.9, where small changes in pH have negligible effect on $\Delta\bar{h}$, which is also sensitive to temperature and ionic strength. $\Delta\bar{h}$ can also be determined experimentally by allowing the reaction to go to completion. Some experimental and determined values are given in Table 1.

RESULTS

At pH 8.4 and I 0.1, the initial velocity, v , was a linear function of the enzyme concentration. Operational Michaelis parameters were determined

Fig. 1. Chromatography of leucine aminopeptidase on diethylaminoethylcellulose. The column (30 cm. $\times$ 2 cm.) was equilibrated with 5 mM-*N*-ethylmorpholine buffer, pH 8.4. Protein (360 mg.) was applied to the column and fractions (5 ml.) were collected at a flow rate of 60 ml./hr. by elution with a concentration gradient of sodium chloride from 0 to 0.2 M (— · — · —). ●, $E_{280 m\mu}$; ○, specific activity.

Fig. 2. Plot of $\Delta\bar{h}$ for the hydrolysis of L-leucylglycine at I 0.1 and 25° by assuming the following apparent pK values: L-leucylglycine, 8.14; L-leucine, 9.64; glycine, 9.67.

Table 1. Values of $\Delta\bar{h}$ for some substrates at ionic strength 0.1 and 25°

Substrate	pH	$\Delta\bar{h}$	
		Calculated	Determined
L-Leucylglycine	8.40	0.538	0.531
	8.66	0.573	0.550
L-Leucylvaline	8.40	—	0.545
L-Leucylalanine	8.40	—	0.526
L-Leucine amide	8.40	0.584	—
L-Leucine benzyl ester	8.40	—	-0.150

Fig. 3. Lineweaver-Burk plots for the hydrolysis of L-leucylvaline at pH 8.4, I 0.1 and 25°. v is the initial velocity of hydrolysis in arbitrary units. O, Enzyme activated in the presence of 5 mM-magnesium chloride; ●, enzyme activated in the presence of 2 mM-manganese chloride.

Fig. 4. Lineweaver-Burk plots for the hydrolysis of L-leucine benzyl ester at pH 8.4, I 0.1 and 25°. The velocities are in the same arbitrary units as Fig. 3 and are corrected for non-enzymic hydrolysis. O, Enzyme activated in the presence of 5 mM-magnesium chloride; ●, enzyme activated by 2 mM-manganese chloride.

by regression of the equation relating $1/v$ and $1/[S]$ (Lineweaver & Burk, 1934), where $[S]$ is the substrate concentration. Some Lineweaver-Burk plots of the equations are shown for L-leucylvaline in Fig. 3 and for L-leucine benzyl ester in Fig. 4. The Michaelis parameters obtained for a number of substrates together with their standard errors are collected in Table 2.

The results of alcohol-inhibition studies are shown in Figs. 5 and 6. For both alcohols studied, the plots for different alcohol concentrations intersect in a point in the top left-hand quadrant. Esters were not produced in these runs in concentrations detectable by paper chromatography.

DISCUSSION

The results clearly show that the pH-stat assay is one of the best methods available for studying the hydrolysis of small peptides. It has been possible for the first time to determine the Michaelis parameters for leucine aminopeptidase (Table 2) and there can be little doubt that similar determinations could be made for other peptidases by the

Fig. 5. Inhibition of leucine aminopeptidase by butan-1-ol at pH 8.4, I 0.1 and 40°. The velocities are in arbitrary units. The concentrations of butanol were: O, 0; ●, 0.073 M; ▲, 0.182 M.

Table 2. Kinetic parameters of leucine aminopeptidase at pH 8.4, I 0.10 and 25°

Substrate	Activation by Mg^{2+} ions		Activation by Mn^{2+} ions	
	K_m (mM)	V_{max}	K_m (mM)	V_{max}
L-Leucine amide	5.21 ± 0.43	0.76 ± 0.07	15.7 ± 4.1	3.38 ± 1.65
L-Leucylglycine	1.00 ± 0.01	0.53 ± 0.01	0.81 ± 0.01	1.54 ± 0.10
L-Leucylvaline	0.51 ± 0.01	0.56 ± 0.03	1.04 ± 0.02	2.34 ± 0.18
L-Leucylalanine	0.79 ± 0.04	0.50 ± 0.02	0.65 ± 0.02	1.53 ± 0.12
L-Leucine benzyl ester	1.56 ± 0.03	0.27 ± 0.01	1.10 ± 0.01	0.42 ± 0.04

Relative values of V_{max} are quoted.

Fig. 6. Inhibition of leucine aminopeptidase by glycerol at pH 8.4, I 0.1 and 25°. The velocities are in arbitrary units. The concentrations of glycerol were: $\circ$, 0; $\bullet$, 0.46 M; $\blacktriangle$, 0.92 M; $\triangle$, 1.38 M.

same method. For all the substrates $V_{\max}$ is greater for the Mn^{2+} ion-activated enzyme than for the Mg^{2+} ion-activated enzyme. With the exception of L-leucine amide, the K_m values for the Mn^{2+} ion-activated and Mg^{2+} ion-activated enzyme are very similar. The results are inadequate to decide whether the kinetics are of the quasi-equilibrium type, and a discussion of the magnitudes of the K_m values would be premature.

It is clear that leucine aminopeptidase has a marked esterase activity with a rate of hydrolysis of L-leucine benzyl ester about one-quarter that of L-leucine amide at high substrate concentrations. Evidence that the same enzyme is involved in esterase and peptidase activities is presented by Bryce & Rabin (1964).

The inhibition by alcohols presents a more complex picture than has been assumed (Hill & Smith, 1957), and is clearly not of the simple competitive type. The simplest scheme of reactions that fits the results is:

This yields an equation of the form:

$$\frac{1}{v} = \frac{1}{[S]} \left(\frac{K_m}{V_{\max}} + \frac{K_m[I]}{K_i V_{\max}} \right) + \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K'_i} \right)$$

The values of K_i and K'_i , calculated from the slopes and intercepts in Figs. 5 and 6, are collected in Table 3. K_i and K'_i are, respectively, the dis-

Table 3. Inhibitor constants for leucine aminopeptidase with glycerol and butanol

Alcohol	K_i	K'_i
Glycerol (0.46 M)	1.2	1.4
Glycerol (0.92 M)	0.9	1.6
Glycerol (1.38 M)	0.8	1.3
Butanol (0.073 M)	0.12	0.26
Butanol (0.182 M)	0.09	0.27

sociation constants for the alcohol complexes of the enzyme and of the enzyme-substrate complex. Alcohols are less firmly bound to the enzyme-substrate complex than to the free enzyme. The marked inhibition by glycerol argues against simple 'hydrophobic interaction' as the major factor in inhibition.

SUMMARY

1. A sensitive precise pH-stat assay for peptidases is described.
2. The Michaelis parameters for a number of substrates of leucine aminopeptidase have been measured.
3. Leucine aminopeptidase hydrolyses L-leucine benzyl ester at one-quarter the rate of L-leucine amide.
4. Leucine aminopeptidase is inhibited by glycerol and butanol, and the dissociation constants of the alcohol complexes of the enzyme and of the enzyme-substrate complex have been computed.

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