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A Peptidase (Aminopeptidase B) from Cat and Guinea Pig Liver Selective for N-Terminal Arginine and Lysine Residues

II. Modifier Characteristics and Kinetic Studies

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Some modifier characteristics and kinetic properties of an aminopeptidase from cat and guinea pig liver were studied and the results compared with those of rat liver aminopeptidase B. The activity of the three enzymes on L-arginine and L-lysine β-naphthylamides was inhibited strongly in the presence of exceedingly small amount of Hg^{2+}, Pb^{2+}, Cd^{2+}, and *p*-chloromercuribenzoate and to a lesser extent by other heavy metal ions. None of the chelating agents used (8-OH-quinoline, EDTA, 1,10-phenanthroline and KCN) significantly inhibited the function of these enzymes. Diethyl *p*-nitrophenylphosphate and di-isopropyl fluorophosphate had no effect. Neither cysteine nor 2-mercaptoethanol activated the enzymes. The most significant effect was the activation by certain monovalent anions, notably chloride ions. The Michaelis constant for the hydrolysis of L-arginine-β-naphthylamide was determined for the three enzymes, as well as the energy of activation for the hydrolysis of L-arginine- and L-lysine-β-naphthylamide. All of the results obtained with the cat and guinea pig preparation were very similar to those obtained with the rat liver enzyme. Therefore, there exists an enzyme in guinea pig and cat liver corresponding to that of rat liver, *i.e.* aminopeptidase B.

In the preceding report the existence of an enzyme was documented in cat and guinea pig liver having similarities to the rat liver aminopeptidase B (APB) described in earlier investigations.[1–3] The notable similarities between the enzymes in these three species were the correspondence in the purification procedures suitable for their purification, their substrate specificity, and the relation of the hydrolysis rates of L-arginine- and L-lysine-β-naphthylamide (L-Arg- and L-Lys-β-NA). In the present investigation further characterization of the enzymes in the various species is presented.

Table 1. The influence of various modifiers on the activity of the *cat*-APB hydrolyzing L-Arg-β-NA. The numbers give the percentage inhibition.

Modifier	Concentration (M)							
	1.66×10^{-3}	0.83×10^{-3}	1.66×10^{-4}	0.83×10^{-4}	1.66×10^{-5}	0.83×10^{-5}	1.66×10^{-6}	1.66×10^{-7}	1.66×10^{-8}
Cysteine	45	35	20	0	0	0	0	0	0
2-Mercaptoethanol	100	75	45	30	0	0	0	0	0
$HgCl_2$	100	100	100	100	100	100	100	100	100
$Pb(NO_3)_2$	100	100	100	100	100	80	80	50	0
$CdCl_2$	100	100	100	100	90	65	45	20	0
$FeCl_2$	100	100	50	20	0	0	0	0	0
$CuCl_2$	100	100	100	100	100	100	60	0	0
$CoCl_2$	60	30	0	0	0	0	0	0	0
$MnCl_2$	50	45	40	10	0	0	0	0	0
PCMB	100	100	100	100	100	100	100	100	100
E-600	0	0	0	0	0	0	0	0	0
DFP	0	0	0	0	0	0	0	0	0
EDTA	100	90	60	40	40	0	0	0	0
8-OH-quinoline	0	0	0	0	0	0	0	0	0
1,10-Phenanthroline	100	100	100	80	10	0	0	0	0
KCN	35	30	25	25	25	20	15	10	00

Table 2. The influence of various modifiers on the activity of the *guinea pig*-APB hydrolyzing L-Arg-β-NA. The numbers give the percentage inhibition.

Modifier	Concentration (M)								
	1.66×10^{-3}	0.83×10^{-3}	1.66×10^{-4}	0.83×10^{-4}	1.66×10^{-5}	0.83×10^{-5}	1.66×10^{-6}	1.66×10^{-7}	1.66×10^{-8}
Cysteine	50	30	25	0	0	0	0	0	0
2-Mercaptoethanol	100	80	60	20	0	0	0	0	0
$HgCl_2$	100	100	100	100	100	100	100	100	100
$Pb(NO_3)_2$	100	100	100	100	100	60	30	20	0
$CdCl_2$	100	100	100	100	100	80	60	40	0
$FeCl_2$	100	100	60	30	0	0	0	0	0
$CuCl_2$	100	100	100	100	100	70	0	0	0
$CoCl_2$	50	20	0	0	0	0	0	0	0
$MnCl_2$	50	40	10	0	0	0	0	0	0
PCMB	100	100	100	100	100	100	100	100	100
E-600	0	0	0	0	0	0	0	0	0
DFP	0	0	0	0	0	0	0	0	0
EDTA	100	90	40	40	30	0	0	0	0
8-OH-quinoline	0	0	0	0	0	0	0	0	0
1,10-Phenathroline	100	100	100	90	10	0	0	0	0
KCN	40	30	20	20	15	0	0	0	0

MATERIAL AND METHODS

The materials and methods were the same as in the previous investigations.[2,3] Any variation in procedure is noted in the detailed description of the experiment. The enzyme preparations used were the same as in the previous papers.[2-5]

RESULTS

1. *The effect of various inhibitors and activators.* The 0.5 ml water normally present in the incubation mixture was substituted by various modifiers at a variety of concentrations. The incubation time was in all cases 30 min, substrate concentration (L-Arg-β-NA) was 0.166 mM and the buffer concentration 0.02 M (phosphate, pH 7.0).

The results are presented partially in Tables 1 and 2 where the figures represent the percentage of APB activity inhibition after a 30 min preincubation with the modifier. Several heavy metals and *p*-chloromercuribenzoate (PCMB) inhibited the hydrolysis of L-Arg-β-NA prominently even in exceedingly small concentrations. It is further noted that the organophosphorus inhibitors had no effect and that the metal chelating agents did not significantly inhibit the reaction. *o*-Phenanthroline was the most effective of these reagents, but its action could be explained as an unspecific anion effect.

Figs. 1—2 show the results obtained when the effect of various monovalent anions was tested on the activity of APB. It is seen that chloride ions cause a remarkable activation and that generally all the anions tested activate the hydrolysis of L-Arg-β-NA in a similar quantitative way in the case of each enzyme; *e.g.*, F^- causes an activation over a wide concentration range in every case. These results are similar to those described for rat liver aminopeptidase B.[3]

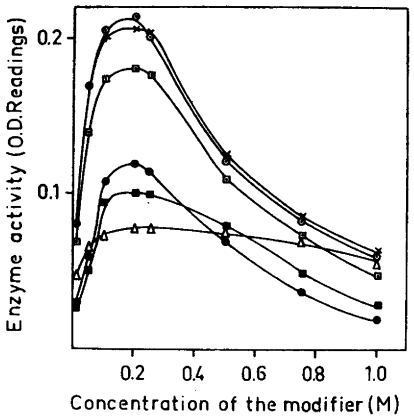

Fig. 1. The effect of various monovalent anions on the *cat* enzyme hydrolyzing L-Arg-β-NA. × = NaCl, ○ = KCl, □ = LiCl, ● = NaBr, △ = NaF and ■ = $NaNO_3$.

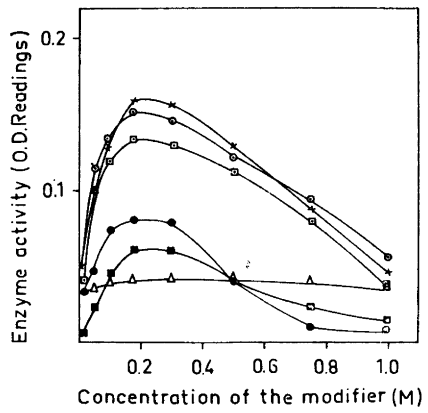

Fig. 2. The effect of various monovalent anions on the *guinea pig* enzyme hydrolyzing L-Arg-β-NA. Explanation of the symbols as in Fig. 1.

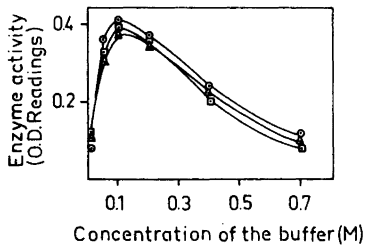

Fig. 3. The effect of TRIS—HCl buffer, pH 7.0, at different concentrations, on the activity of the three different enzyme preparations. □ = *guinea pig*, ○ = cat, and △ = rat.

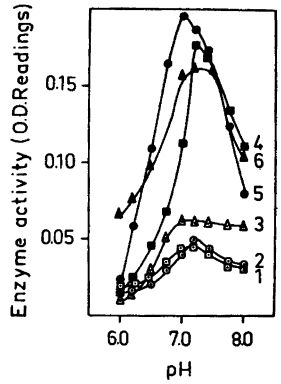

Fig. 4. The activity of the three different enzyme preparations in 0.01 M phosphate buffer in the absence [curves 1 (*guinea pig*, □), 2 (cat, ○) and 3 (rat, △)] and in the presence of 0.2 M NaCl [curves 4 (*guinea pig*, ■), 5 (cat, ●) and 6 (rat, ▲)].

Fig. 3 represents the behaviour of these three enzyme preparations in TRIS—HCl buffer at varied concentrations. The optimal concentration of buffer for all of the enzymes is 0.1—0.2 M. Figs. 4—6 show the effect of added NaCl (0.2 M) in some other buffers. It is seen that all of the enzymes are activated strongly in the presence of chloride ions.

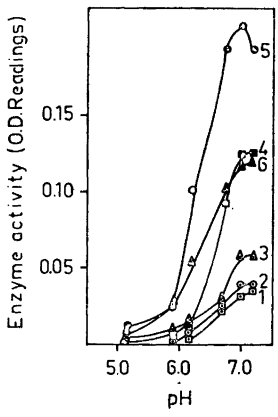

Fig. 5. The activity of the three different enzyme preparations in 0.01 M, β-dimethylglutaric acid buffer. Explanation of the symbols as in Fig. 4.

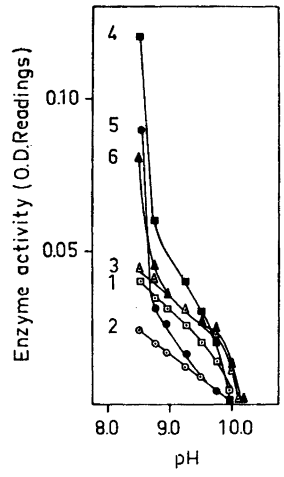

Fig. 6. The activity of the three different enzyme preparations in 0.01 M glycine-NaOH buffer. Explanation of the symbols as in Fig. 4.

2. *Photo-oxidation.* In these experiments the enzymes were diluted with 0.1 M TRIS—HCl buffer, pH 7.0, in which was dissolved methylene blue (MB) in a concentration in the final reaction mixture (2.5 ml) of a) 10^{-5} M and b) 10^{-6} M. The mixture was composed, consequently, of 1.5 ml 0.1 M TRIS—HCl buffer, pH 7.0, 0.5 ml enzyme preparation in 0.1 M TRIS—HCl buffer, pH 7.0, containing MB, and 0.5 ml water.

The mixture was left in test tubes under the influence of direct sunlight or light from an Osram incandescent lamp (400 W, at 20 cm distance) in a glass-walled tube in ice (4° to 6°C) for various times (0—30 min). Afterward the substrate (0.5 ml β-Arg-β-NA) at a final concentration of 0.166 mM was added and the solutions incubated in the usual way for 60 min at 37°C under normal room lighting. Simultaneously mixtures containing all components but MB as well as the above solutions in the absence of strong light (under normal room lighting) were treated in the same way.

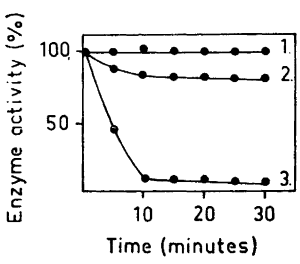

Fig. 7. The effect of photo-oxidation with methylene blue on the cat liver enzyme. Curve 1: radiation without MB; Curve 2: radiation with 1 μM MB; Curve 3: radiation with 10 μM MB.

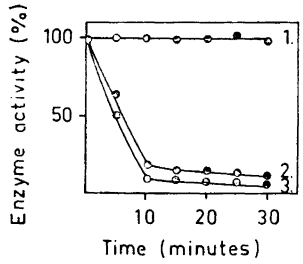

Fig. 8. The effect of photo-oxidation with methylene blue on the guinea pig liver enzyme. Curve 1: radiation without MB; Curve 2: radiation with 1 μM MB; Curve 3: radiation with 10 μM MB.

The results are shown in Figs. 7—9. It is seen that radiation destroys the enzyme activity in the presence of even small amounts of MB. The strong radiation and MB were both necessary for this loss of activity. MB without radiation had no effect nor had radiation without MB. In addition MB at the concentration used in these experiments did not affect the reaction between β-naphthylamine and fast Garnet GBC (employed in the determination of APB activity). There was a slight difference in the results obtained when using the Osram lamp to replace direct sunlight. The effect of direct sunlight seemed to be about 10 % greater. Figs. 7—9 represent the results obtained in sunlight.

3. *Substrate affinity.* The Michaelis constant (K_m) was determined for the three enzyme preparations with L-Arg-β-NA. The constants were determined by the usual Lineweaver-Burk method and the results drawn as the double reciprocal plot for L-Arg-β-NA (Fig. 10).

The values of K_m for the cat and rat liver enzymes were 0.11 mM and 0.10 mM, respectively, but the K_m for the guinea pig enzyme was about twice

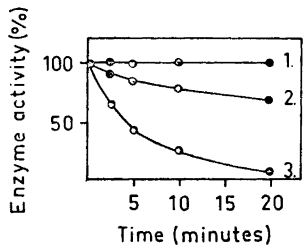

Fig. 9. The effect of photo-oxidation with methylene blue on the rat liver APB. Curve 1: radiation without MB; curve 2: radiation with 1 μM MB; Curve 3: radiation with 10 μM MB.

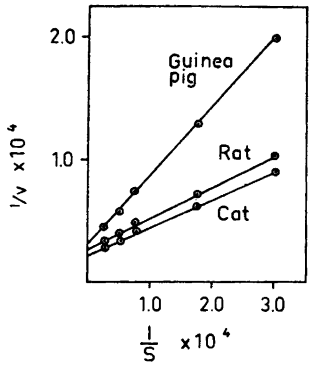

Fig. 10. A plot of reciprocal velocity *versus* reciprocal substrate concentration for the activity of the three different enzyme preparations on L-Arg-β-NA for the determination of K_m. Expressed in M and hydrolysis rate in Klett-Summerson readings. K_m = 0.11 mM (cat), 0.20 mM (*guinea pig*) and 0.10 mM (rat).

as large, *i.e.* 0.20 mM. Generally, the K_m values of other peptidases have about the same order of magnitude.

4. *Energy of activation.* The energy of activation for the enzyme catalyzing the hydrolysis of L-Arg- and L-Lys-β-NA was determined. In these experiments the substrate concentration employed was ten times the K_m value (L-Arg-β-NA) so that the velocity might approach V_{max}. The reaction velocity was measured at 21, 32, and 37°C in the case of the cat and guinea pig preparation, and at 7, 22.5, and 32°C when using the rat enzyme. Figs. 11—13 are

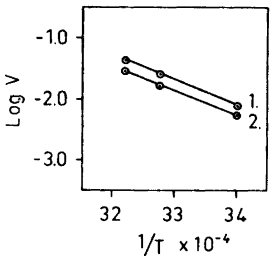

Fig. 11. A plot of $\log_{10}$ of velocity *versus* the reciprocal of absolute temperature for the activity of the *cat* liver enzyme on L-Arg-β-NA and L-Lys-β-NA for the determination of the energy of activation, E = 17 500 cal mole^{-1}. Curve 1: L-Arg-β-NA, Curve 2: L-Lys-β-NA as substrate.

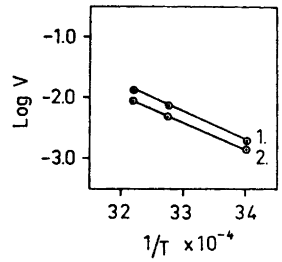

Fig. 12. A plot of $\log_{10}$ of velocity *versus* reciprocal temperature for the activity of the *guinea pig* liver enzyme on L-Arg-β-NA and L-Lys-β-NA for the determination of the energy of activation. E = 20 000 cal mole^{-1}. Curve 1: L-Arg-β-NA, Curve 2: L-Lys-β-NA as substrate.

the plots of the logarithm of the velocity *versus* the reciprocal of the absolute temperature in the case of these three enzymes using the above two substrates.

The following values were obtained for the energy of activation with both of the substrates: cat 17 500, guinea pig 20 000 and rat 18 500 cal mole^{-1}.

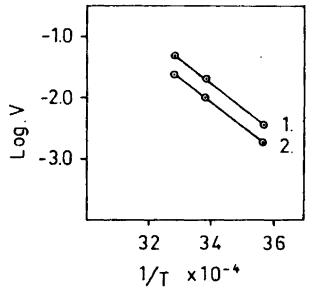

Fig. 13. A plot of $\log_{10}$ of velocity *versus* reciprocal temperature for the activity of the *rat* liver enzyme on L-Arg-β-NA and L-Lys-β-NA for the determination of the energy of activation. $E = 18\,500$ cal mole^{-1}. Curve 1: L-Arg-β-NA, curve 2: L-Lys-β-NA as substrate.

These results are not in close agreement with the values reported for other peptide bond-cleaving enzymes, the energy of activation usually being somewhat smaller (about 10 000 or less).

DISCUSSION

The present and the previous investigations demonstrate that an aminopeptidase, similar to that found in rat liver, occurs also in cat and guinea pig liver. This conclusion is based on purification of the enzymes from the livers of all of these species and on a comparison of several of their characteristics. The subjects of comparison included substrate specificity, effect of activators and inhibitors, effect of various buffers, the values of the Michaelis constant, *etc.* with a marked similarity of findings in all of the species studied. In addition, the energy of activation (E) of the hydrolysis of both the basic amino acid β-naphthylamides and the rapid inactivation of the enzyme by photo-oxidation in the presence of methylene blue disclosed further similarities.

The three enzyme preparations had nearly the same energy of activation for the hydrolysis of the two substrates utilized, *i.e.* close to 20 000 cal mole^{-1}. Both L-Arg- and L-Lys-β-NA offered the same E value in each preparation studied, indicating that the energy of activation appears to be more characteristic for the enzyme than for the substrate. The fact that all these preparations had similar values for E, suggests that they might have a similar functional mechanism. The fact that such high energies of activation were obtained may signify that the relative number of collisions between the enzyme and the substrate leading to hydrolysis of the substrate is small in the case of these enzymes.

The effect of photo-oxidation on the enzyme was tested in order to clarify the nature of the active center of the aminopeptidases in question. It is known that histidine, methionine, tryptophan, and tyrosine are photo-oxidized in the presence of MB.[6] This fact has been utilized in studies of the structure of the active center of various enzymes.[7] The concentrations of MB used in such

experiments have usually been higher than in this study. Therefore it is of special interest that the APB-activity was strongly reduced even in the presence of 10^{-6} M MB. It would appear that these enzymes have at least some of the above aminoacids at or near the active center.

As to the modifier characteristics, there seem to be several notable points:

1. Heavy metal ions caused a loss of enzyme activity when present even in very small concentrations. Such a behaviour may indicate the presence of an essential SH-group at or near the active center, though this effect can also be due to the denaturating properties of the ions. It is also known that N-terminal imidazole has a great affinity for heavy metal ions.[7]

2. None of the metal chelating agents caused a clear inhibition, so that these enzymes are apparently not metal-dependant. This is also supported by the fact that none of the divalent metal ions tested activated the enzymes.

3. Because the organophosphorus compounds tested had no effect on the enzymes, it is likely that the enzymes have no serine residues at the active centre.

4. The effect of the monovalent anions tested was the most remarkable finding. The chloride ions caused so clear an effect that it would be natural to think these enzymes were specifically activated by them.

The aim of this and the previous report was to demonstrate the possible similarity of the partially purified hepatic enzymes. The few differences which occurred might be due to differences in the purification. The properties of the cat, quinea pig, and rat liver enzymes investigated are apparently so similar that the existence of a rat APB-like enzyme in the other mammalian species is concluded.

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