THE HYDROLYSIS OF AMINO ACYL-β-NAPHTHYLAMIDES BY PLASMA AMINOPEPTIDASES

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Historically, the study of aminopeptidase activity in mammalian systems has been directed primarily toward tissue extracts. This work culminated in the isolation and characterization of the classical leucine aminopeptidase (Smith and Hill, 1960). Little attempt has been made to characterize the aminopeptidases of normal plasma and serum, and perhaps it is for this reason that the activity has been tacitly identified with tissue leucine aminopeptidase (LAP), and commonly designated serum LAP. Behal et al. (1963) have chromatographically resolved the LAP activity of human blood into a number of aminopeptidase components.

In this report, some distinguishing properties are described for a variety of aminopeptidases which can occur in blood plasma. The aminopeptidase activity of normal plasma could not be attributed to the presence of leucine aminopeptidase.

Methods. The blood which was drawn from laboratory animals with siliconized equipment was anticoagulated with one tenth volume of cold 0.1 M ammonium oxalate, chilled, and centrifuged at 1,500 x g for 30 min. in order to obtain platelet-poor plasma. Plasma was prepared in such a way as to minimize the possibility of contamination with erythrocyte (Haschen, 1961) and platelet (Balogh, 1963) aminopeptidases.

The conventional assay mixture contained the plasma sample and the appropriate amino acyl-β-naphthylamide substrate (5×10^{-4} M) in 0.0625 M Tris-HCl, pH 7.4, vol. 0.4 ml, temp. 37 C. The reaction rates were followed by means of a continuous recording adaptation of a fluorometric aminopeptidase assay (Greenberg, 1962). The recorded rates were corrected for the fluorescence and quenching characteristics of the reaction mixture, and for the anticoagulant content of the plasma.

Results. As described in Table 1, purified hog kidney LAP (Worthington) hydrolyzed the β-naphthylamide (β-NA) substrates with the same relative affinities as one would predict on the basis of the known reaction rates for the enzyme on the corresponding amino acid

amide substrates (Smith and Hill, 1960). In addition, LAP hydrolyzed L-leucine-β-NA with a pH optimum near 8.5; the reaction was competitively inhibited by L-leucinamide and L-methioninamide; and Mg^{++} enhanced the reaction rate while EDTA and oxalate were inhibitory.

Table 1. Rates of Hydrolysis of Amino Acyl-β-Naphthylamides by Assorted Plasma Preparations
μmole (10^{-3})/min/ml of plasma

Source	-β-naphthylamide						
	Leu	Met	Phe	Arg	Tyr	Lys	Ala
Leucine aminopeptidase †	(100)	(55)	(17)	(7)	(6)	(6)	(2)
Human	20	25	26	11	22	7	44
	(100)	(125)	(130)	(55)	(110)	(35)	(220)
Rhesus monkey	43	58	68	27	60		88
	(100)	(135)	(158)	(63)	(140)		(205)
Fischer rat	17	40	21	6.7	16	2	30
	(100)	(236)	(124)	(39)	(94)	(12)	(177)
CCl_4-treated rat	90	134	80			21	90
	(100)	(149)	(89)			(23)	(100)
CCl_4-treated rat (+SH)	112	194	131			106	202
	(100)	(173)	(117)			(95)	(180)
Tumor rat	17	26	14	7.7	12	5.8	24
	(100)	(153)	(82)	(45)	(71)	(34)	(140)
Tumor rat (+SH)	38	57	51	48	27	42	67
	(100)	(150)	(134)	(126)	(71)	(110)	(175)

The reaction mixture contained 25 μliters of plasma, 5×10^{-4} M -β-naphthylamide, 0.0625 M Tris-HCl, pH 7.4, vol. 0.4 ml, temp. 37 C. For SH activation, 2-mercaptoethanol was incorporated at 0.01 M.

CCl_4-treated rats were gavaged 5 times, at 2-day intervals, with 0.05 ml CCl_4. Tumor rat blood was taken 7 weeks after implantation of pituitary tumor cells into a hind leg of a Fischer rat.

† Figures within parentheses represent the reaction rates relative to 100 for the rate of L-Leu-β-NA hydrolysis. Leucine aminopeptidase was a Worthington preparation.

As illustrated in Table 1, the aminopeptidase activity of rat, monkey, and human blood plasma showed a very different order of specificity on the amino acyl-β-NA substrates as compared with LAP. The rates, which appeared to be independent of fasting considerations, showed a pH optimum near 7.2 on all the β-NA substrates tested. The

rate of L-Leu-β-NA hydrolysis by human plasma was essentially the same as reported for the hydrolysis of L-Leucylglycine by normal human serum (Fleisher, et al., 1958), however, as recorded in Table 1, plasma aminopeptidase(s) showed even greater activity on the β-NA derivatives of L-alanine, L-methionine and L-phenylalanine. Rat plasma showed its greatest rate of hydrolysis on L-Met-β-NA, whereas human and monkey plasma favored L-Ala-β-NA. However, after the addition of Co^{++} to the reaction mixture human plasma hydrolyzed these two substrates at essentially the same rate (Figure 2). The latter observation is in contrast with the very low rate of L-Ala-β-NA hydrolysis by LAP (Table 1).

The aminopeptidase nature of the plasma activity was indicated by its lack of action on the N-benzoyl amino acyl-β-NA substrates, and by the competitive inhibition exhibited by amino acid amides. The hydrolysis of L-Met-β-NA by rat plasma was inhibited by amino acid amides: Met·NH_2 > Leu·NH_2 > Arg.NH_2, whereas N-benzoyl-L-Met·NH_2 exhibited no inhibition. The relative inhibitory nature of the amides was in agreement with the relative substrate specificities shown by the plasma aminopeptidase(s) for the β-naphthylamides.

The aminopeptidase(s) of normal plasma, as is the case for LAP, showed neither an activation by thiol compounds nor a reversible inhibition with thiol reagents. However, the plasma aminopeptidase activity of Fischer rats bearing an (F4) ACTH-secreting, pituitary tumor (Furth, et al., 1957) showed a pronounced thiol activation, as well as an 80% inhibition with p-chloromercuriphenyl sulfonate (10^{-4} M). This inhibition could be reversed with 2-mercaptoethanol. As shown in Table 1, the addition of 2-mercaptoethanol (10^{-2} M) to the assay mixture produced a profound alteration of both the absolute and relative rates of hydrolysis. Plasma from tumor rats showed the greatest rates of hydrolysis on L-Arg-β-NA. This thiol-activated plasma aminopeptidase of the tumor-bearing rats is probably identical to the thiol-activated aminopeptidase which has been observed in pituitary extracts (Ellis, 1963).

Puromycin, while it exhibited no inhibition of LAP, was found to be an effective inhibitor of the thiol-activated aminopeptidase occuring in the normal pituitary (Ellis, 1964). When puromycin was utilized in the present study it became possible to further discriminate between the plasma aminopeptidase(s) and LAP. Whereas puromycin did not alter the rate of hydrolysis of L-Leu-β-NA by LAP, this same antibiotic was found to inhibit the hydrolysis, by human plasma, of all the substrates shown in Table 1. The amount of inhibition of the plasma aminopeptidase(s) provided by 6×10^{-4} M puromycin ranged from 56% for L-Lys-β-NA to 92% for L-Phe-β-NA. Purified LAP was unaffected by 6×10^{-4} M puromycin.

Plasma prepared from CCl_4-treated rats showed an elevated rate of hydrolysis of L-Leu-β-NA (Table 1), and a decreased puromycin sensitivity. Such activity could be attributed to leucine aminopeptidase. On the other hand, the hydrolysis of L-Lys-β-NA showed a marked (5 fold) activation by 2-mercaptoethanol, and this activity was very significantly inhibited (> 90%) by puromycin. The latter activity could not be attributed to LAP, but rather to a thiol-activated aminopeptidase which appeared to serve as a more sensitive indicator of experimental tissue injury than did leucine aminopeptidase.

Figure 1 provided some indication for the existence in the plasma of aminopeptidase inhibitors, and emphasized the importance of plasma dilution when determining relative reaction rates. Essentially the same dilution effects were observed for rat, monkey, and human plasma aminopeptidase activities. A study of rate response to enzyme concentration (under zero order conditions) showed that it was necessary to accomplish a plasma dilution of 1/16, or more, in the assay mixture in order to achieve a linear rate response on all the substrates tested. Inhibitory factors were implicated since dialysis of the plasma samples provided for an increased rate response in the presence of Co^{++} (even for L-Ala-β-NA), and since K_m values were found to vary inversely with plasma dilution. Reliable K_m values could not be extrapolated.

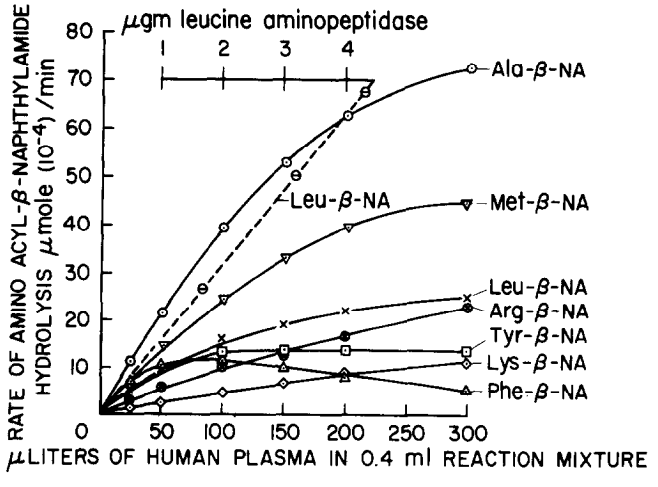

Figure 1. The reaction mixture: 5 x 10 M β-naphthylamide substrate, 0.0625 M Tris-HCl, pH 7.4, temp. 37 C. The broken line shows a linear rate response for purified leucine aminopeptidase (Worthington) on L-Leu-β-NA substrate.

There was detectable hydrolysis of L-Gly-β-NA and L-Try-β-NA, but not so for the β-naphthylamides of L-Val, L-His, L-Pro, L-Ser, L-Thr, L-Cys, γ-L-Glu, and α-L-Glu.

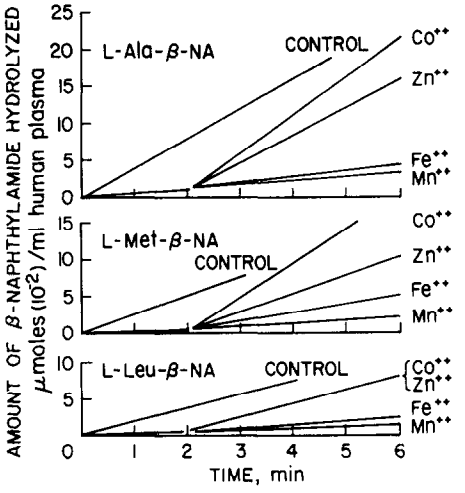

Figure 2. The reaction mixture: 50 μliters of dialyzed plasma, 5 x 10^{-4} M β-naphthylamide substrate, 0.0625 M Tris-HCl, pH 7.4, vol. 0.4 ml, temp. 37 C. Two minutes after the reaction was initiated, metal was added to give 10^{-4}M. The reaction rates, which were taken from recorder tracings, are compared with the control rates obtained with undialyzed plasma. Dialysis was conducted 24 hours against 400 volumes of 0.15 M NaCl-0.01 M EDTA-5 x 10^{-3} M Tris-HCl, pH 7.4, temp. 3 C. A second 24 hour dialysis was conducted, but with the omission of EDTA from the dialyzing medium.

Although Mn^{++} has been reported to be the best activator for LAP (Smith and Spackman, 1955), it appeared that similar findings had not been reported for normal "serum LAP". To the contrary, it had been observed that Mn^{++} provided relatively little activation for the hydrolysis of L-leucylglycine by serum LAP, whereas Co^{++} showed considerable activation (Fleisher, 1954). Metal activation studies with dialyzed human plasma described in Figure 2, showed that Mn^{++} was incapable of restoring the aminopeptidase-catalyzed hydrolysis of L-Ala-β-NA, L-Met-β-NA, or L-Leu-β-NA. On the other hand, Zn^{++} gave full restoration of activity, and Co^{++} gave restoration of rates which were equal to, or in excess of, the normal aminopeptidase activity of undialyzed plasma. With respect to LAP, Zn^{++} and Co^{++} are reported to be inhibitory and ineffective, respectively (Smith and Hill, 1960).

In summary, it appeared that a variety of aminopeptidases can occur in blood plasma. At least three major aminopeptidase types could be distinguished in plasma: (1) The aminopeptidase(s) of normal plasma, which was predominantly thiol-independent, Co^{++}-activated, and

puromycin-sensitive. L-Ala-β-NA and L-Met-β-NA were the substrates most readily hydrolyzed by whole plasma. The results implicated more than one aminopeptidase in normal plasma. (2) The thiol-activated aminopeptidase(s), which appeared in plasma in association with tissue injury, was thiol-dependent, Co^{++}- and Mn^{++}-activated, and puromycin-sensitive. L-Lys-β-NA and L-Arg-β-NA were the preferred substrates. (3) Leucine aminopeptidase, which was only detectable in plasma in the event of tissue injury, was thiol-independent, Mn^{++}-activated and puromycin-insensitive. L-Leu-β-NA was the substrate most readily hydrolyzed.

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