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α-*N*-Benzoylarginine-2-naphthylamide Hydrolase (Cathepsin B1?) from Rat Skin. III. Substrate Specificity, Modifier Characteristics, and Transformation of the Enzyme at Acidic pH

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Some properties of rat skin benzoylarginine-2-naphthylamide hydrolase types I (preparations I and AI) and II (preparations II and NII) were studied. Both types were activated by dithiothreitol and EDTA, but responded differently to 1 mM KCN, when benzoylarginine-2-naphthylamide (BANA) was used as a substrate: type I was inhibited, while type II was activated. When leucine-2-naphthylamide was used as a substrate, both types were activated by KCN. Thiol proteinase inhibiting substances, like heavy metals, iodoacetic acid, 4-chloromercuribenzoic acid, and tosyllysine chloromethylketone, inhibited the enzymes. Diisopropylfluorophosphate, phenylmethylsulfonylfluoride, 4-aminobenzamidine, and high-molecular-weight trypsin inhibitors were without effect. The substrate specificity of rat skin BANA hydrolase resembled that of an amino acid naphthylamidase, naphthylamides of methionine, lysine, arginine, and alanine being hydrolyzed most rapidly. The rate of hydrolysis of BANA was only 11 % of that of methionine naphthylamide. Amino acid esters with a free α-amino group were also good substrates. The transformation of type II to type I at acidic pH was studied. During the transformation amino acids or peptides were formed and probably some inhibitor present in type II was destroyed proteolytically.

In a previous report the purification of the rat skin α-*N*-benzoylarginine-2-naphthylamide hydrolyzing enzyme was presented.[1] Two enzyme types, preparations I and II, were separated by DEAE cellulose chromatography. The specific activity of preparation II increased markedly, when its concentration in the reaction mixture was lowered by dilution, while dilution had no effect on the specific activity of preparation I. Treatment of preparation II at pH 4 and 55 °C increased the activity of the undiluted enzyme 4.5 times and the effect of dilution on the specific activity of the enzyme was lost. The isoelectric point and chromatographic behavior of acid treated preparation II were similar to those of preparation I suggesting that a transformation of enzyme type II to type I had occurred as a consequence of the acid treatment. In this report some characteristics of the transformation of type II to type I as well as substrate specificity and modifier characteristics of the enzyme are presented.

MATERIALS AND METHODS

Enzyme preparations. Rat skin BANA hydrolase preparations (I, AI, II, and NII) were purified as described previously.[1]

Substrates. α-*N*-Benzoyl-DL-arginine-2-naphthylamide hydrochloride (BANA) and α-*N*-benzoyl-L-arginine-4-nitroanilide hydrochloride (BAPA) were obtained from Fluka A.G.; α-*N*-benzoyl-DL-arginine amide hydrochloride hydrate (BAA) was from Merck A.G.; L-alanine-2-naphthylamide hydrobromide (Ala-NA), L-arginine-2-naphthylamide (Arg-NA), and L-lysine-2-naphthylamide carbonate (Lys-NA) were from Nutritional Biochemicals Corp.: 1-L-glutamic acid-2-naphthylamide (Glu-NA), L-methionine-2-naphthylamide (Met-NA), and α-*N*-tosyl-L-arginine-2-naphthylamide (Tos-Arg-NA) were from Schwarz/Mann, and L-arginine methylester (Arg-ME), *N*-acetyl-L-tyrosine ethylester (ATEE), α-*N*-benzoyl-L-arginine methylester (BAME), bovine hemoglobin (Hb), and L-leucine-2-naphthylamide (Leu-NA) from Sigma Chem. Co.

Modifiers. Bovine lung trypsin-kallikrein inhibitor (Tracylol^R, 10.000 kallikrein inhibiting

equivalents (KIE)/ml) was obtained from Bayer A.G.; dithiothreitol (DTT) from Calbiochem; diisopropylfluorophosphate (DFP) and 4-chloromercuribenzoic acid were from Fluka A.G.; benzethonium chloride and phenylmethylsulfonylfluoride (PMSF) from Schwarz/Mann; 4-aminobenzamidine, *N*-ethylmaleimide, iodoacetic acid, iodoacetamide, puromycin, α-*N*-*p*-tosyl-L-lysine chloromethylketone hydrochloride (TLCK), ovomucoid trypsin inhibitor (type II-O), and soybean trypsin inhibitor (SBTI, type II-S) were from Sigma Chem. Co.

Activity determinations. The hydrolysis rates of naphthylamides, amides, 4-nitroanilides, esters, and proteins were determined as described in previous reports.[1,2] In modifier experiments with metal ions the Britton-Robinson buffer[2] was replaced with a Tris-maleate buffer,[3] pH 5.8, containing 0.6 mM DTT and 3 mM KCN. All modifiers were dissolved in the assay buffers and preincubated for 15 min at room temperature with the enzyme solution. Undiluted enzyme preparations were used unless otherwise stated.

Isolation of peptides formed during acid activation of preparation II. Preparation II (20 ml) was dialyzed against 5 mM ammonium formate buffer, pH 4.0, at +4 °C. The solution was ultrafiltrated with a Diaflo [R] 8-MC apparatus using a UM-10 membrane (Amicon N. V., Holland) and 10 ml of the filtrate was collected as a control. The volume of the enzyme solution was adjusted to 20 ml with the ammonium formate buffer, and the enzyme solution incubated for 20 min at 55 °C. The enzyme solution was again ultrafiltrated and 10 ml of the filtrate collected. The filtrates were then lyophilized (the ammonium formate buffer being evaporated during the lyophilization). The dry residues were dissolved in 1 ml of 50 % (*v*/*v*) methanol and the amino acids in the solutions were determined with ninhydrin, using L-leucine (Sigma Chem. Co.) as a standard.[4] Samples of filtrates were pipetted onto silica gel thin layer plates (Kieselgel G, Merck A. G.) and ascending chromatography was performed with a 1-buthanol-acetic acid-water solvent system (60:20:20 *v*:*v*:*v*), and stained with ninhydrin.[5]

RESULTS

Activation of BANA hydrolase with thiol activators. The effects of DTT, EDTA, and KCN were tested on purified enzyme preparations I, AI, and NII using BANA and Leu-NA as substrates (Fig. 1). DTT activated the hydrolysis of both substrates by all enzyme preparations, maximally at concentrations over 0.1 mM. EDTA activated the enzyme preparations only slightly, which indicated that heavy metals were not present in the enzyme solutions. Enzyme types I and II responded oppositely

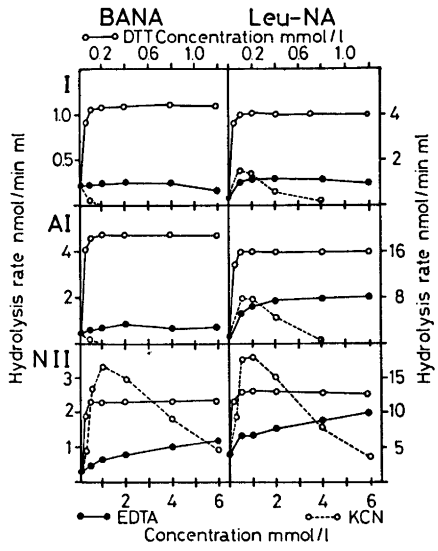

Fig. 1. Effects of DTT, EDTA, and KCN on the Leu-NA and BANA hydrolyzing activities of enzyme preparations I, AI, and NII.

on KCN, when BANA was used as a substrate; type I was inhibited and type II activated by 1 mM KCN. On the other hand, both types were activated by KCN, when Leu-NA was used as a substrate. KCN caused similar

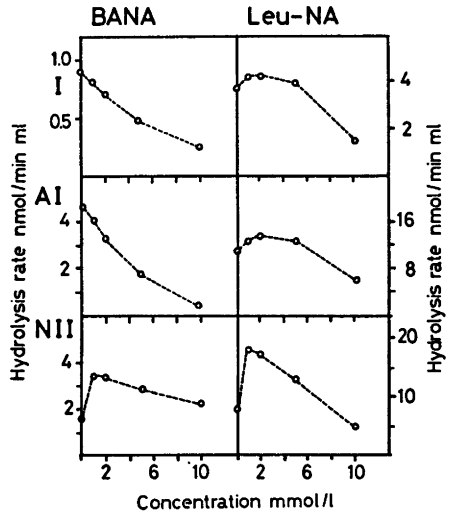

Fig. 2. Effect of KCN on the Leu-NA and BANA hydrolyzing activities of purified preparations I, AI, and NII, when the reaction mixtures contained DTT (0.2 mM) and EDTA (1 mM).

Table 1. Effects of some inhibitors on the activity of BANA hydrolase. TM = Tris-maleate buffer, BR = Britton-Robinson buffer, KIE = kallikrein inhibiting equivalent (see "Materials and Methods").

Inhibitor	Conc. mM	Buffer	Relative activities of preparations I	AI	NII
Control		BR, TM	100	100	100
Pb-acetate	1	TM	0	0	0
$CuCl_2$	1	TM	0	0	0
$HgCl_2$	1	TM	0	0	0
$CoCl_2$	1	TM	0	0	0
$FeCl_2$	1	TM	10	10	20
$CdCl_2$	1	TM	0	0	0
$CaCl_2$	1	TM	80	80	90
$ZnCl_2$	1	TM	0	0	0
$MnCl_2$	1	TM	50	50	60
$MgCl_2$	1	TM	90	90	90
Iodoacetic acid	1	BR	0	0	5
Iodoacetamide	1	BR	10	10	5
4-Chloromercuribenzoic acid	0.1	BR	20	20	20
N-Ethylmaleimide	1	BR	10	10	30
TLCK	0.1	BR	0	0	0
4-Aminobenzamidine	1	BR	100	100	100
Benzethonium chloride	1	BR	150	150	160
Puromycin	1	BR	70	70	70
DFP	1	BR	100	100	100
PMSF	1	BR	100	100	100
SBTI	0.1[a]	BR	104	100	100
Tracylol[R]	16.6[b]	BR	110	110	110
Ovomucoid	0.1[a]	BR	100	100	100

[a] mg/ml. [b] KIE/ml.

effects, when the reaction mixture contained optimal concentrations of DTT and EDTA (0.2 and 1 mM, Fig. 2), but higher concentrations of KCN (> 10 mM) were needed for total inhibition of the BANA hydrolyzing activity of type I.

Inhibitors. In Table 1 the effects of some inhibitors on the hydrolysis of BANA by preparations I, AI, and NII are presented. Heavy metal ions Pb^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+}, Fe^{2+}, Cd^{2+}, and Zn^{2+} were found to be strong inhibitors, while Mg^{2+}, Mn^{2+}, and Ca^{2+} were only slightly inhibitory. Tosyllysine chloromethylketone, iodoacetic acid, iodoacetamide, 4-chloromercuribenzoic acid, and *N*-ethylmaleimide strongly inhibited all BANA hydrolase preparations. The high-molecular-weight trypsin inhibitors from soybean, ovomucoid, and bovine lung (Tracylol [R]) were without effect on the activity, while puromycin exerted a slight inhibition. Benzethonium chloride caused a slight activation at 1 – 5 mM concentrations, but inhibited BANA hydrolase preparations at higher concentrations (over 10 mM). NaCl (0.01 – 0.20 M) and methanol (1 – 10 %) had no effects on the enzyme activities.

Substrate specificity. In Table 2 relative hydrolysis rates of various substrates in comparison to the hydrolysis rate of BANA are presented. The BANA hydrolase preparations hydrolyzed naphthylamides, nitroanilides, amides, and esters of various amino acids. The most readily hydrolyzed substrates were Met-NA, Arg-ME, Lys-NA, Arg-NA, and Ala-NA, in decreasing order. Substrates with a free α-amino group were hydrolyzed faster than corresponding substrates with an acylated α-amino group: Arg-NA 4.7 times faster than BANA and Arg-ME 8.7 times faster than BAME. Nitroanilides were hydrolyzed slower than corresponding naphthylamides: BAPA was not hydrolyzed at all and the hydrolysis rate of Leu-PA was 50 – 65 % of that of Leu-NA, at pH 5.8. Tos-Arg-NA was not hydrolyzed,

Table 2. Substrate specificity of rat skin BANA hydrolase. Relative hydrolysis rates, in comparison to that of BANA, are given. The values are expressed as the means of the activities of the enzyme preparations from three separate purification procedures. The specific activities of the preparations were (nmol min^{-1} mg^{-1}): preparation I, 49.3, 77.1, and 71.3; preparation AI, 394, 495, and 460; and preparation NII, 68.6, 99.3, and 95.2.

Substrate	Concentration Substr. mM	Methanol % (v/v)	pH	Relative activities of preparations I	AI	NII
BANA	1.66	6.66	5.8	100	100	100
Met-NA	1.66	6.66	5.8	790	740	920
Lys-NA	1.66	6.66	5.8	540	590	590
Arg-NA	1.66	6.66	5.8	470	470	490
Ala-NA	1.66	6.66	5.8	540	550	500
Leu-NA	1.66	6.66	5.8	240	260	450
Leu-NA	1.66	6.66	7.0	390	340	510
Val-NA	1.66	6.66	5.8	210	250	280
Glu-NA	1.66	6.66	5.8	160	150	180
Tos-Arg-NA	1.66	6.66	5.8	0	0	0
Leu-PA	0.33	6.66	5.8	160	130	210
BAPA	0.33	6.66	5.8	0	0	0
BAA	20	0	5.8	30	40	10
Arg-ME	3.33	0	5.8	760	780	670
TEE	3.33	0	5.8	360	400	230
BAME	3.33	0	5.8	90	90	90
ATEE	3.33	0	5.8	10	10	5
Hemoglobin	0.66[a]	0	4.3	0	0	80

[a] %.

and the rate of hydrolysis of BAA by preparations I and AI was 30 – 40 %, and by preparation NII 10 % of that of BANA. The relative activity of BAA/BANA always increased when preparation II was pretreated at pH 4 and 55 °C. Hemoglobin was hydrolyzed by preparation NII, but not by preparations I and AI. The hemoglobin hydrolyzing activity was not inhibited by iodoacetic acid or TLCK, suggesting that a cathepsin D contaminant was present in preparation NII.

The substrate specificities of preparations I and AI (type I) were similar, while some differences in their specificities in comparison to that of preparation NII (type II) was noticed. The relative activity (as compared to the hydrolysis rate of BANA) of type II was higher than that of type I, when amino acid naphthylamides were used as substrates, while the relative activity of type I was higher than that of type II, when BAA or amino acid esters were used as substrates.

The Michaelis constants of preparations I, AI, and NII were determined for BANA by the Lineweaver-Burk method. A Michaelis constant of 1.4 mM was obtained for preparations I and AI, and 5.0 mM for preparation NII. BANA inhibited the hydrolysis of Leu-NA by preparation NII, at pH 5.8. The specific activity of the undiluted enzyme was 9.4 nmol min^{-1} mg^{-1}, when BANA was used as a substrate, and 40.9 nmol min^{-1} mg^{-1}, when Leu-NA was used as a substrate. If BANA and Leu-NA were simultaneously in the reaction mixture, liberation of naphtylamine was only 30.1 nmol min^{-1} mg^{-1}.

Transformation of enzyme type II to type I. When enzyme type II (preparation II) was preincubated at pH 4 and 55 °C, the activity of the undiluted enzyme was increased and in a subsequent DEAE cellulose chromatography the enzyme was eluted as type I (preparation AI).[1] In Fig. 3 the dependence of this activation on pH and temperature is presented, using a fixed incubation time (20 min). At pH 7.5 the enzyme was labile at 45 °C. At pH 4.5, the activity increased by increasing temperature up to 60 °C, and at pH 4.0 the maximal activity was reached at 55 – 60 °C. At more acidic pH's (3.5 – 3.0) the maximal activity was

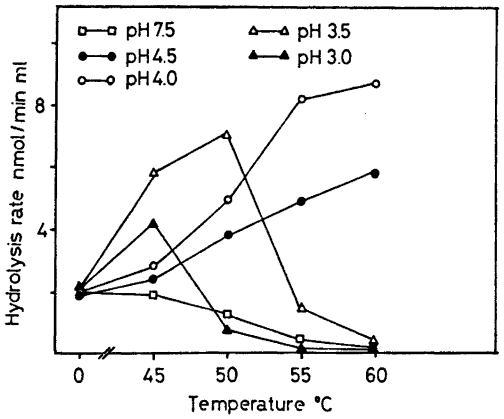

Fig. 3. Effect of preincubation of preparation II at various pH's and temperatures, for 20 min, on the BANA hydrolase activity of the undiluted enzyme.

obtained at lower temperatures (50 – 45 °C), and higher temperatures destroyed the enzyme. In Fig. 4 the activation effects of preincubation times at a fixed temperature (55 °C) and different pH's are presented. At pH 7.5 the activity of the enzyme was destroyed within 30 min. At acidic pH's (5.0 or below) activation of BANA hydrolase was noticed. The initial rate of the activation reaction increased with increasing acidity of the solution, but at pH's below 4.0 the activated enzyme was labile at 55 °C. At pH 4.0 the activity reached a plateau within 10 min and remained unchanged during the next 20 min. The most reproducible activation of preparation II was obtained by using an incubation time of 20 min, at pH 4.0

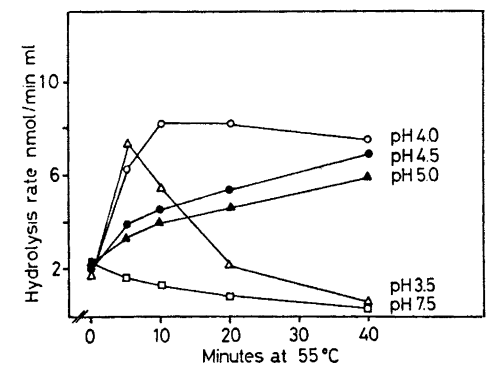

Fig. 4. Effect of preincubation time at various pH's, at 55 °C on the BANA hydrolyzing activity of preparation II.

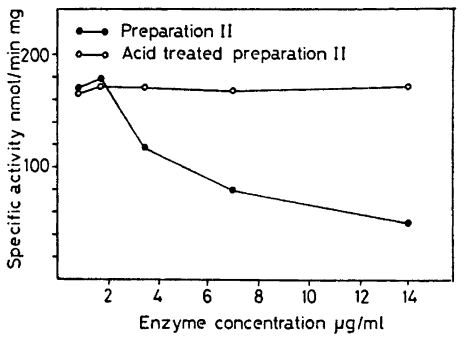

Fig. 5. Effect of enzyme concentration on the specific activity of preparation II and acid treated preparation II.

and 55 °C. After acid activation, the specific activity of preparation II did not increase by diluting the enzyme solution. In Fig. 5 the specific activities of preparation II and acid activated preparation II as a function of enzyme concentration are presented. It is interesting to notice that the specific activities of the acid treated and nontreated enzymes approach each other at low enzyme concentrations.

Since the activation of various proteolytic enzymes is a proteolytic reaction itself an investigation was made on the protease activity of preparation II, at pH 4.0 and 55 °C. When BANA or Leu-NA were added to the activation mixture at a final concentration of 1.66 mM, no formation of naphthylamine was observed. However, when hemoglobin, at a final concentration of 0.66 %, was incubated with preparation II at pH 4.0 and 55 °C, a marked hydrolysis occurred.

The role of an autolytic protease in the activation of preparation II at pH 4, at 55 °C, was studied by isolating possible peptides formed during the reaction, by an ultrafiltration technique (details are given in "Materials and Methods"). During the incubation of preparation II (20 ml) at pH 4, 55 °C, the total activity of the undiluted enzyme increased from 52 nmol/min to 218 nmol/min, with a concomitant release of 40.1 nmol of amino acids. A thin-layer chromatography of isolated peptides or amino acids is presented in Fig. 6.

Distinct spots of amino acids or peptides were found in the ultrafiltrate of the acid treated preparation II, the most prominent spot having an R_F value similar to that of glycine

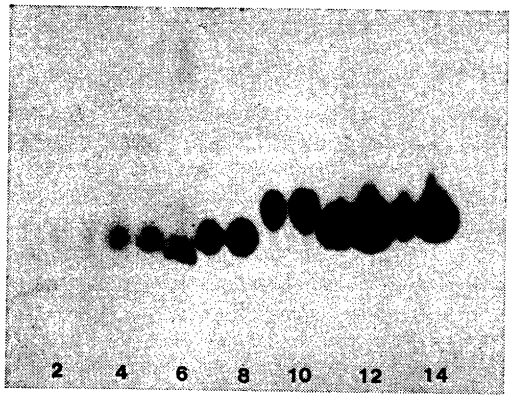

Fig. 6. A thin layer chromatography of ultrafiltrates of preparation II and acid-treated preparation II. 1 – 3, ultrafiltrates of preparation II; 4 – 6, ultrafiltrates of acid treated preparation II (10, 20, and 40 μl); 7 – 8, glycine; 9 – 10, glutamic acid; 11 – 12, serine; 13 – 14, threonine (5 and 10 μg).

(0.40), while the ultrafiltrate of the untreated preparation II contained only very little ninhydrin positive material. The experiments suggest that transformation of type II to type I is a proteolytic reaction.

DISCUSSION

The rat skin BANA hydrolase preparations are, like cathepsin B1 isolated from various tissues, activated by thiols (DTT) and chelating reagent (EDTA). Activation by EDTA was not very marked while DTT strongly activated the enzyme. BANA hydrolase types I and II responded oppositely to 1 mM KCN, when BANA was used as a substrate; type I was inhibited and type II activated. When Leu-NA was used as a substrate, both types were activated by KCN. The actual reason for different responses of enzyme types I and II to KCN remained unsolved, as did the differences in the effects of KCN on the hydrolysis of Leu-NA and BANA, by enzyme type I. In the literature, the reported effect of KCN on the activity of cathepsin B1 varies.[6–8] It seems that various cathepsin B1 like enzymes respond differently to KCN, as do rat skin BANA hydrolase types I and II.

In inhibitor experiments, the rat skin BANA hydrolase preparations behaved as expected for thiol-activated proteinases. Serine proteases acylating inhibitors and high-molecular-weight trypsin inhibitors were not inhibitory. 4-Aminobenzamidine, a potent competitive inhibitor of trypsin [9] did not inhibit, and puromycin, which inhibits a pituitary thiol-activated arylamidase,[10] inhibited BANA hydrolase preparations only slightly. Benzethonium chloride, which inhibits some salivary arylamidases [11] and cathepsin B1 of beef spleen,[12] activated rat skin BANA hydrolase preparations at low concentrations (up to 5 mM), but caused inhibition at higher concentrations.

The substrate specificity of rat skin BANA hydrolases differed markedly from the specificities of cathepsin B1 preparations of various tissues. Substrates with a free α-amino group were hydrolyzed much faster than substrates with a blocked α-amino group. This was evident with both naphthylamide and ester substrates, and is in contrast with the specificity of most known cathepsin B1 preparations, which do not hydrolyze substrates with a free α-amino group.[7,8,12–16] However, a highly purified cathepsin B1 preparation from bovine spleen hydrolyzes Leu-NA.[17] The following facts suggest that rat skin BANA hydrolase also hydrolyzes Leu-NA:

1. The BANA and Leu-NA hydrolyzing activities were not separated by salt precipitation, gel chromatography, ion exchange chromatography, isoelectric focusing, or polyacrylamide gel electrophoresis.[1]

2. Acid treatment of type II enzyme at pH 4 and 55 °C caused similar changes in the properties of BANA and Leu-NA hydrolyzing activities (with the exception of the activation by KCN).

3. BANA inhibited the hydrolysis of Leu-NA by preparation NII.

Besides BANA, the rat skin enzyme preparations also hydrolyzed BAA, but BAPA was not hydrolyzed at all. The deficiency in the hydrolysis of BAPA by cathepsin B1 preparations has also been noticed in rat liver [16] and sheep thyroid gland.[15] Preparations II and NII hydrolyzed hemoglobin. This activity was not inhibited by iodoacetate or TLCK, which suggested that a hemoglobin hydrolyzing cathepsin D contaminate was present in preparations II and NII.[18] Cathepsin B1 is known to be a powerfull proteinase [8,12,14,19–21] and this proteolytic activi-

ty distinguishes it from rat skin BANA hydrolase.

BANA hydrolase of the rat skin behaved like an aminopeptidase-like arylamidase. Thiol-activated amino acid naphthylamidases have been found in rat skin,[22,23] testis,[24] kidney,[25,26] and liver.[26] The naphthylamidase from rat kidney and liver lysosomes [25,26] has properties very similar to those of skin BANA hydrolase. The lysosomal naphthylamidase is active optimally at pH 7.0, and is activated by dithiothreitol.[26] The enzyme preferentially hydrolyzes the naphthylamides of arginine, lysine, phenylalanine, and leucine, and blocking of the α-amino group of Arg-NA with a carbobenzoxy group lowers the hydrolysis rate by 76 %. It is, however, known that the lysosomal amino acid naphthylamidase differs from the BANA hydrolase purified from rat liver lysosomes.[16]

Treatment of BANA hydrolase type II at pH 4 and 55 °C for 20 min resulted in formation of type I with concomitant changes in isoelectric point, chromatographic behavior,[1] and effect of KCN on BANA hydrolyzing activity of the enzyme. During the acid treatment amino acids or peptides were formed and any added hemoglobin was hydrolyzed, while BANA and Leu-NA remained unchanged. These facts suggest that the activation at acidic pH is a proteolytic reaction probably catalyzed by an acidic protease, cathepsic D, present in type II enzyme preparations.[18] The molecular modification may take place in the BANA hydrolase molecule itself, or through some inhibitor associated with it. The fact that the specific activity of type II increased by dilution, while the specific activity of type I was uneffected suggests the latter mechanism. However, an inhibitor destroyed proteolytically must be a comparatively small molecule, since no difference in molecular sizes of types I and II was noticed by gel filtration.[1] Such a small inhibitor has been isolated from mouse haptoglobin.[27] The rat skin inhibitors of BANA hydrolase demonstrated in the previous report [1] have molecular weights of about 74 000 and 13 000, and the dissociation of these inhibitors from BANA hydrolase should cause a positive change in the molecular weight of the enzyme.

Slight activation of cathepsin B1 isolated from various tissue extracts has been noticed after the extracts were autolyzed at pH 3 – 4.[8,20,28] The reported activation is comparatively small, 20 – 30 %, while rat skin BANA hydrolase was activated 400 %. Barret [8] also reports a two-fold activation of purified human liver cathepsin B1, after storage of the enzyme at pH 5.0, in the presence of EDTA, at +4 °C. The isoelectric point of the human liver enzyme was not changed during storage, suggesting that the mechanism of the activation of the human liver enzyme differs from that of the rat skin BANA hydrolase. No activation of the rat liver lysosomal BANA hydrolase has been noticed, after the enzyme had been incubated at pH's below 5.0.[16] The rat skin BANA hydrolase is, like cathepsin B1 preparations of various tissues, readily inactivated at alkaline pH and elevated temperatures.[8,12,16]

α-*N*-Benzoylarginine-2-naphthylamide has widely been used as a substrate of cathepsin B1. However, Distelmaier *et al.*[29] have shown that testing of the BANA hydrolyzing activity of a tissue extract may lead to erroneous conclusions on the presence of cathepsin B1 in the tissue. Rat skin BANA hydrolase also has many properties that cannot be attributed to cathepsin B1 and the BANA hydrolyzing activity of the skin seems to be associated with a thiol-activated arylamidase.

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