Specificity in the hydrolysis of N-acyl-L-phenylalanine 4-nitroanilides by chymotrypsin

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Summary. The affinity of N-acyl-L-phenylalanine 4-nitroanilides for chymotrypsin is enhanced as the hydrophobicity of non-amino acid residues in the P_2-position of the substrates increases, whereas k_{cat} remains nearly constant. On the other hand, if alanine or leucine is in the P_2-position k_{cat} increases with decreasing K_M.

The binding of specific peptide substrates to chymotrypsin occurs by hydrophobic interactions in the so-called tosyl pocket[2] as well as by subsite interactions [3-7]. The inactivation of chymotrypsin by peptidyl chloromethyl ketones in concert with X-ray crystallographic studies[8,9] reveals that there are, besides hydrogen bonds, additional hydrophobic interactions of the amino acid residue in the P_2-position (nomenclature according to Schechter and Berger[10] of the inhibitor with the subsite S_2 of the enzyme. The P_2-S_2 contact is also documented by the enzymic hydrolysis of oligopeptide amides[6,11] and denatured proteins[12].

To get more information on the influence of hydrophobic N-acyl residues in chymotrypsin substrates, we have studied the hydrolysis of differently substituted L-phenylalanine 4-nitroanilides by chymotrypsin. Glt-, Glt-εAhx-, Glt-(εAhx)$_2$-, Glt-Ala-, and Glt-Leu- were used as N-acyl residues.

Material and methods. Bovine α-chymotrypsin (chymotrypsin A_4) was purchased from Boehringer, Mannheim. The content of active sites (89%) in the protein was determined by titration with 4-nitrophenyl acetate[13]. The protein concentration was calculated by the use of $A^{1\%}_{280} = = 20.0$.

Preparation of the substrates. Glt-Phe-Nan was synthesized according to Erlanger et al.[14]. The preparation of Glt-εAhx-Phe-Nan and Glt-(εAhx)$_2$-Phe-Nan has been described earlier[15]. Glt-Ala-Phe-Nan, m.p. 180–183 °C, $[\alpha]^{22}_D$ −10.0° (c = 1, methanol) and Glt-Leu-Phe-Nan, m.p. 178–179 °C, $[\alpha]^{22}_D$ −9.0° (c = 1, methanol) were synthesized from Boc-Ala-Phe-Nan, m.p. 167–170 °C, $[\alpha]^{21}_D$ −16.4° (c = 1, ethyl acetate) and Boc-Leu-Phe-Nan, m.p. 98–99 °C, $[\alpha]^{21}_D$ −13.7° (c = 1, ethyl acetate) by removal of the Boc-groups and subsequent acylation according to the procedure used for Glt-Phe-Nan.

All elemental analyses were within acceptable limits. TLC also indicated homogeneity [chloroform–methanol (7:1), n-butanol–acetic acid–water (4:1:1), isobutanol–formic acid–water (75:13.5:11.5)]. The degree of enzymic hydrolysis of all substrates was > 97%.

Kinetic measurements. All measurements were carried out in 0.05 M Tris · HCl buffer, 0.05 M $CaCl_2$, 1% (v/v) dimethylformamide, pH 7.8, at 25 °C using a Perkin-Elmer M 356 or a Beckman DK-2A spectrophotometer. Enzymic hydrolysis was followed by the increase in absorption of the liberated 4-nitroaniline at 405 nm (ε = 9800 M^{-1} cm^{-1}) and 390 nm (ε = 11,300 M^{-1} cm^{-1}). K_M and k_{cat} were calculated from the slopes and intercepts of the Lineweaver-Burk and Eadie plots. 5–7 runs were performed for each determination of K_M and k_{cat}.

The direct determination of the 2nd-order rate constant k_{cat}/K_M was carried out under pseudo 1st-order conditions. The conditions were fulfilled by $[S] \leq 0.1\ K_M$ and $[E] \leq 0.01\ K_M$. k_{cat}/K_M was calculated from the pseudo 1st-order rate constant $k_{cat} \cdot [E]/K_M$.

All data were obtained by 3 independent measurements. The standard deviations of the constants were 7–12%.

Results and discussion. The hydrolysis of ester and amide substrates by chymotrypsin can be described as follows[4]:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA + P_1 \xrightarrow{k_3} E + P_2$$

For amides and anilides, acylation is the rate-limiting step, i.e. $k_{cat} = k_2$ and $K_M = K_s$[4].

Kinetic parameters listed in the table show that there are significant differences in the hydrolysis between the substrates containing either Ac, Glt, and εAhx or L-amino acid residues (Ala, Leu) in the P_2-position. In the series of Ac-, Glt-, Glt-εAhx-, and Glt-(εAhx)$_2$-Phe-Nan K_M decreases as the hydrophobicity of the N-acyl residues increases, whereas k_{cat} remains nearly constant. In contrast to the substrates mentioned, k_{cat} increases with decreasing K_M values in the hydrolysis of Glt-Ala- and Glt-Leu-Phe-Nan. The K_M values of Glt-Ala- and Glt-Leu-Phe-Nan are higher than the corresponding values of the substrates without an α-amino acid residue in P_2. On the other hand, 19-fold and 64-fold k_{cat} values were found for Glt-Ala- and Glt-Leu-Phe-Nan compared with Glt-εAhx-Phe-Nan.

The high k_{cat} values of Glt-Ala- and Glt-Leu-Phe-Nan can be explained by specific enzyme-substrate interactions, which bring the scissile bond into a favourable position to the catalytic residues of the enzyme. X-ray studies of Aγ-chymotrypsin modified by N-acetyl tripeptide chloromethyl ketones indicate 3 hydrogen bonds between enzyme and inhibitor[8]. 2 of these hydrogen bonds (between NH of P_1 and CO of Ser-214; CO of P_3 and NH of Gly-216) can be formed in the binding of Glt-Ala(Leu)-Phe-Nan. The hydrophobic interactions between P_2 and S_2 were confirmed by the inactivation rate of chymotrypsin by Ac-Leu-Phe-CH_2Cl that was 2.9 times faster than that by Ac-Ala-Phe-CH_2Cl[9]. Similarly, we found that the ratio of the k_{cat} values of Glt-Leu-Phe-Nan and Glt-Ala-Phe-Nan amounts to 3.3.

In the chymotrypsin-catalyzed hydrolysis of peptide amides, which contain an aliphatic α-amino acid residue in P_2, k_{cat} increases with decreasing K_M[6,11]. Despite the low K_M values the k_{cat} values of Glt-(εAhx)$_{1,2}$-Phe-Nan are much lower than the k_{cat} values of Glt-Ala(Leu)-Phe-Nan. This behaviour of Glt-(εAhx)$_{1,2}$-Phe-Nan in the enzymic hydrolysis can be interpreted as follows. The low K_M values should

Kinetic parameters for the α-chymotrypsin-catalyzed hydrolysis of N-acyl-L-phenylalanine 4-nitroanilides

Substrate	[S] (mM)	K_M (mM)	k_{cat} (sec^{-1})	k_{cat}/K_M ($M^{-1} \cdot sec^{-1}$)
Ac-Phe-Nan*	-	0.40	0.01	(25)
Glt-Phe-Nan	0.10–1.0	0.25	0.021	84 (96)
Glt-Phe-Nan**	-	0.28	0.013	46
Glt-εAhx-Phe-Nan	0.04–0.8	0.056	0.02	350
Glt-(εAhx)$_2$-Phe-Nan	0.01–0.5	0.016	0.019	1180
Glt-Ala-Phe-Nan	0.10–2.0	1.00	0.38	380 (418)
Glt-Leu-Phe-Nan	0.08–0.8	0.50	1.28	2560 (2680)

Measurements were carried out in 0.05 M Tris · HCl buffer, 0.05 M $CaCl_2$, pH 7.8, containing 1% (v/v) dimethylformamide, at 25 °C. The enzyme concentration varied in the range from 1 to 10 µM. The k_{cat}/K_M values in parentheses were determined directly under pseudo 1st-order conditions. For details see methods'section. * The values were taken from Petkov et al.[16] and ** from Erlanger et al.[14].

result from hydrophobic interactions of the Glt-(εAhx)$_{1,2}$-residues with S_2 and/or other apolar sites[9] on the protein surface. However, this contact does not lead to an acceleration of catalysis due to the failure of the hydrogen bond between CO of P_3 and NH of Gly-216. In the series of Ac-, Glt-, Glt-(εAhx)$_{1,2}$-Phe-Nan K_M decreases, whereas k_{cat} is nearly constant. Therefore, non-productive binding of the N-acyl residues should not be important, since such interactions lower both K_M and k_{cat}[17,18]. In addition, non-productive binding of the aniline moiety could not be substantiated[19].

The high affinity of Glt-(εAhx)$_{1,2}$-Phe-Nan to the enzyme is reflected in the reaction rate under pseudo 1st-order conditions ($[S] \ll K_M$). Thus, the k_{cat}/K_M value of Glt-(εAhx)$_2$-Phe-Nan is 3 times higher than that of Glt-Ala-Phe-Nan. In contrast, the k_{cat} values of Glt-Ala-Phe-Nan is 20 times higher than the k_{cat} value of Glt-(εAhx)$_2$-Phe-Nan.

The k_{cat} and k_{cat}/K_M values in the table show that Glt-Leu-Phe-Nan is more specific than the other substrates studied. Glt-Leu-Phe-Nan should also be of practical interest as a chromophoric chymotrypsin substrate because it is hydrolyzed much faster than the frequently used Glt-Phe-Nan.

1 Abbreviations: Ac-, acetyl-; εAhx-, 6-aminohexanoyl-; Boc-, tert.-butyloxycarbonyl-; Glt-, glutaryl-; Nan-, 4-nitroanilide.
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C-banding pattern on the chromosomes of the Japanese house shrew, *Suncus murinus riukiuanus*, and its implication

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Summary. The C-band on the chromosomes of the Japanese house shrew, *Suncus murinus riukiuanus* (Insectivora), was studied. Various types of C-banding pattern were found in the genome of this subspecies. Such banding patterns could be useful for an understanding of autosome and sex-chromosome polymorphisms within *S. murinus*.

Karyotypes of the house shrew, *Suncus murinus*, occurring in various areas of Asia have already been studied, and autosomal and sex chromosomal polymorphisms have been found in this species[3-7], but information on the C-band of the house shrew has as yet only been obtained for the Indian taxon[7]. Nevertheless, C-band staining is a good tool for examining polymorphisms. In this paper, we report the C-banding pattern for the genome of the Japanese house shrew, *S. m. riukiuanus*, and also present its conventional data again here, because the karyotype figure has not been published in detail.

Materials and methods. 3 male specimens collected from Naha, Okinawa Is., Prefecture of Okinawa, were investigated karyologically[8]. The C-band treatment followed the method of Sumner[9], and both conventional staining and C-band treatment were performed on the same preparation. For chromosomal classification the method of Patton was adopted[10].

Results. The C-banding patterns on the autosomes of *S. m. riukiuanus* are as follows. Within the M · SM-elements (row 1, figure 1), a large pair has a centromeric C-band, which is difficult to detect (see also figure 2A); a small pair and a medium-sized one have a distinct centromeric heterochromatic region; and another small pair exhibits terminal C-bands on its short arm, but does not have centromeric C-band. 2 pairs of ST-autosomes are C-band negative. Of the A-autosomes (row 2 and 3, figure 1), only 5 pairs having a short arm were C-band positive; the remaining 8 pairs seem to be devoid of demonstrable C-band material. 4 pairs of the former A-autosomes have both centromeric C-bands and heterochromatic short arms, but a medium-sized pair (placed in the 2nd position of the 2nd row) is distinguished by a lesser amount of C-banded material than is present in the other 3 pairs. On the other hand, the smallest acrocentric pair (5th in the 2nd row), has a terminal C-band only.

The C-banding pattern on the sex chromosomes (figures 1 and 2, B and C) differs markedly from that of the autosomes. The SM-X chromosome has a broad C-band covering the distal one-third of the long arm and a small terminal C-band on the short arm. The SM-Y chromosome

Lengths of the X and Y chromosomes relative to the female haploid set in the Japanese house shrew, *Suncus murinus riukiuanus*, calculated from 20 metaphase plates

	X		Y	
	Mean	SD	Mean	SD
Relative length	10.49 (5.81)	0.72 (0.35)	5.02	0.60

Values in parentheses show the relative length of only the euchromatic portion of the X chromosome.