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Activities of Anionic and Cationic Trypsins in the Temperature Range from 5 to 37°C. Mutant Anionic Trypsins as a Model of Cold-Adapted (Psychrophilic) Enzymes

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Abstract—Temperature dependences of kinetic constants (k_{cat} and K_m) were studied for enzymatic hydrolysis of N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-arginine-*p*-nitroanilide and N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-lysine-*p*-nitroanilide by bovine cationic and rat anionic (wild-type and mutant) trypsins. The findings were compared with the corresponding literature data for hydrolysis of N-benzoyl-DL-arginine-*p*-nitroanilide by bovine cationic trypsin and natural trypsins of cold-adapted fishes. The anionic and cationic trypsins were found to differ in organization of the S_1-substrate-binding pocket. The difference in the binding of lysine and arginine residues to this site (S_1) was also displayed by opposite temperature dependences of hydrolysis constants for the corresponding substrates by the anionic and cationic trypsins. The data suggest that the effect of any factor on the binding of substrates (the K_m value) to the anionic and cationic trypsins and on the catalytic activity k_{cat} should be compared only with the corresponding data for the natural enzyme of the same type. Mutants of rat anionic trypsin at residues K188 or Y228 were prepared by site-directed mutagenesis as approximate models of natural psychrophilic trypsins. Substitution of the charged lysine residue in position 188 by hydrophobic phenylalanine residue shifted the pH optimum of the resulting mutant trypsin K188F from 8.0 to 9.0-10.0, similarly to the case of some natural psychrophilic trypsins, and also 1.5-fold increased its catalytic activity at low temperatures as compared to the wild-type enzyme.

Key words: anionic and cationic trypsins, psychrophilic trypsins, mutant trypsins, temperature dependence

Most animal species have genes encoding both cationic and anionic trypsins. The cause of such variety is unknown. Isoelectric points of these two enzymes differ by six pH units. As a rule, amino acid sequences of cationic trypsins of different organisms are more alike than sequences of anionic trypsins of the same species, and vice versa. Therefore, it was suggested that the trypsin-encoding gene had been duplicated during early stages of evolution, at least before the origin of fishes [1, 2].

Three-dimensional structures of the bovine cationic and rat anionic trypsins are very alike, the greatest difference being found in the amino acid sequence and correspondingly in the structural organization of regions of the surface loops surrounding the substrate-binding site [3]. Especially amino acid substitutions in loop 2 (residues 221-228) can influence the binding of substrates; therefore, cationic and anionic trypsins are suggested to differ in the secondary substrate specificities [1, 4].

The interest in studies on enzymatic activities of the rat wild-type anionic trypsin and its mutant at residues 188 and 228 at 5-10°C was aroused by unusual features of trypsins from fishes living at these temperatures and also by the theoretical and practical importance of elucidation of structural and functional bases of enzyme adaptation to low temperatures (psychrophilicity).

Comparative characteristics of homologous enzymes from psychrophilic, mesophilic, and thermophilic organisms show that their specific activities are, as a rule, similar under conditions of the temperature optimum. Thus, efficiencies of psychrophilic enzymes are very high. At present, the molecular basis of this phenomenon is unknown. The comparison of the primary sequences of

Abbreviations: BAPNA) N-benzoyl-DL-arginine-*p*-nitroanilide; N-Suc-Ala-Ala-Pro-Arg-*pNA*) N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-arginine-*p*-nitroanilide; N-Suc-Ala-Ala-Pro-Lys-*pNA*) N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-lysine-*p*-nitroanilide.

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homologous enzymes suggests a high conservativeness of amino acid residues of their active sites and substrate-binding regions. This finding together with data on the noticeably decreased thermostability of enzymes from psychrophiles became a basis for a hypothesis that explained the high specific activity of these enzymes by an increased flexibility of their molecule [5]. However, correlation between the stability and activity of psychrophilic enzymes is not unambiguously established, and there are some findings [6, 7] contradicting this hypothesis.

Catalytic activities of most of known enzymes adapted to low temperature ("cold" enzymes) are increased mainly due to increased values of k_{cat}, whereas the efficiency (k_{cat}/K_m) of psychrophilic trypsins isolated from fishes living at low temperatures (*Gadus morhua*, *Paranotothenia magellanica* Forster, *Salmo gairdneri*, etc.) is mainly contributed by decreased values of K_m [8-11]. Based on structural analysis of amino acid sequences and X-ray data for psychrophilic and mesophilic trypsins, Smalas et al. [5] suggested that the increased affinity of "cold" trypsins for substrate should be due to increase in the local mobility of the S_1-binding pocket region on substitution in molecules of mammalian trypsins by hydrophobic amino acid residues of residues involved in formation of hydrogen bonds and also of some charged amino acid residues. Moreover, such substitutions are suggested to decrease the electrostatic potential in the S_1-binding pocket that also promotes the increased affinity for substrates.

In connection with this hypothesis, it seems that mutant anionic trypsins with the positively charged K188 residue substituted by a hydrophobic one (K188F, K188Y, K188W, and K188H) and also Y228F unable to produce the hydrogen bond may be used as approximate models of "cold" trypsins. The present study was also motivated by the finding that all fish psychrophilic trypsins studied are anionic, although it is not known whether it is the necessary structural condition. However, it is worth attention that the only known fish cationic trypsin isolated from Atlantic salmon together with several anionic trypsins has no features of a psychrophilic enzyme, unlike the latter, but its properties are similar to those of mammalian trypsin [1].

However, all temperature dependences reported for kinetic constants of psychrophilic trypsins [8-10] were obtained with the bovine cationic trypsin used as the control mesophilic enzyme. The temperature dependences of the corresponding constants for such anionic trypsins are unknown. All mutant trypsins used in the present study were anionic; the temperature dependences of kinetic constants of their hydrolysis were compared with the corresponding data for both the initial rat anionic trypsin and bovine cationic trypsin.

Moreover, the pH optimums of various fish psychrophilic trypsins fishes were shifted from 8.0 to 9.5-10.8 [8, 11, 12]. But because of insufficient knowledge about basic structural and functional dependences of cold adaptation, these specific features seem to deserve investigation, especially because some mutant trypsins also have the activity optimum shifted to alkalinity.

MATERIALS AND METHODS

The bovine trypsin (TPCK-treated, 10,000-13,000 units/mg) was from Sigma (USA); the substrates N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-arginine-*p*-nitroanilide (N-Suc-Ala-Ala-Pro-Arg-*pNA*) and N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-lysine-*p*-nitroanilide (N-Suc-Ala-Ala-Pro-Lys-*pNA*) were from Bachem (Germany); Tris and $CaCl_2$ were from Merck (Germany).

Mutant trypsins were prepared by oligonucleotide-directed mutagenesis according to Kunkel [13] using one-chain uracil-containing DNA templates as described earlier [14]. The rat wild-type anionic trypsin and the corresponding mutants K188F, K188Y, K188W, K188H, and Y228F were secreted into the periplasm of *E. coli* and purified by chromatography on a column with CM-Sepharose (fast-flow) and by affinity chromatography on benzamidine-Sepharose as described in [15]. Concentration of the active enzyme was determined by titration of the active site [14-17].

Kinetic determinations. Hydrolysis of the substrates was followed using a Varian Cary 13E spectrophotometer (USA) recording production of free *p*-nitroaniline at 405 nm (ε = 10,400). To determine the initial rates of hydrolysis, each substrate was used in twelve concentrations in the range suitable for determination of the kinetic constants: 10-60 μM for the arginine-containing substrate (the higher concentrations could not be used because of noticeable inhibition with the substrate) and 50-500 μM for the lysine-containing substrate. Value of ΔA/min was determined by initial slopes of the kinetic curves. Michaelis–Menten parameters were calculated by Eadie–Hofstee plots. The hydrolysis was performed at 37, 20, 10, and 5°C in 50 mM Tris-HCl buffer (pH 8.0) supplemented with 20 mM $CaCl_2$.

RESULTS AND DISCUSSION

Absence of thermal inactivation. Highly effective substrates used in the study allowed us to easily determine the initial rates of their hydrolysis by trypsin and its mutants within a short time, no more than 10-15 min. Therefore, no thermal inactivation of the enzyme occurred, and the present study dealt only with kinetic properties of the different trypsins. To prove the absence of thermal inactivation, the control sample of the enzyme was incubated at the temperature studied during the time required for the experiment.

Temperature dependence of the catalytic constant (k_{cat}) and Michaelis constant (K_m) of anionic and cationic trypsins. Activities of psychrophilic trypsins and bovine cationic trypsin were determined earlier [8-11] using short synthetic amide and ester substrates: methyl ester of N-tosyl-L-arginine and N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). In the present study for the same purpose highly effective substrates were used, such as *p*-nitroanilides of N-protected tetrapeptides: N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-arginine-*p*-nitroanilide (N-Suc-Ala-Ala-Pro-Arg-*pNA*) and N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-lysine-*p*-nitroanilide (N-Suc-Ala-Ala-Pro-Lys-*pNA*). Unlike the binding of N-substituted amino acid derivatives, not only the interaction of P_1-residues of arginine and lysine with the S_1-site of the enzyme should be evident on the binding of such extended substrates, but also secondary interactions that is more like the modeling of proteolysis.

Values of k_{cat} for these highly effective substrates are tens of inverse seconds, whereas the rate of trypsin hydrolysis of BAPNA is much lower (k_{cat} ≈ tens of inverse minutes). Therefore, to compare our findings with the literature data, we used the temperature dependences of relative values of kinetic constants, with the k_{cat} value for each substrate at 37°C taken as 100%.

The temperature dependences of the k_{cat} value for hydrolysis of both arginine- and lysine-containing extended substrates by the rat anionic trypsin were found to be described by the same exponential curve. Moreover, not only the corresponding temperature dependences of k_{cat} relative values of mutant trypsins, but also of fish psychrophilic trypsins (in the latter case this dependence for BAPNA as the substrate was calculated from data presented in [10]) was also similar to this exponential curve (Fig. 1, plot *1*). The apparent activation energy E_a determined from the Arrhenius dependence (logk_{cat} versus 1/T) of hydrolysis of these three substrates by different anionic trypsins was in the range of 7,000-10,000 cal/mol (Table 1). The similarly calculated temperature dependences of k_{cat} relative values for hydrolysis of the same three substrates (in the case of BAPNA from data of the work [10]) by the bovine cationic trypsin, in spite of the greater dependence on the substrate, could also be described by the same exponential dependence (Fig. 1, plot *2*), with E_a in the range of 13,000-20,000 cal/mol (Table 1). Thus, the E_a values of these two isoenzymes were different, with E_a of the cationic form nearly twofold higher. Consequently, the lower activation energy was specific for all anionic trypsins, independently of their psychro- or mesophilicity. The literature data on thermodynamic characteristics of psychrophilic trypsins of different fishes compared to bovine trypsin are somewhat inconsistent [9, 10]. However, considering the findings of the present work, it is obvious that anionic "cold" trypsins should not be compared to bovine cationic trypsin by this parameter.

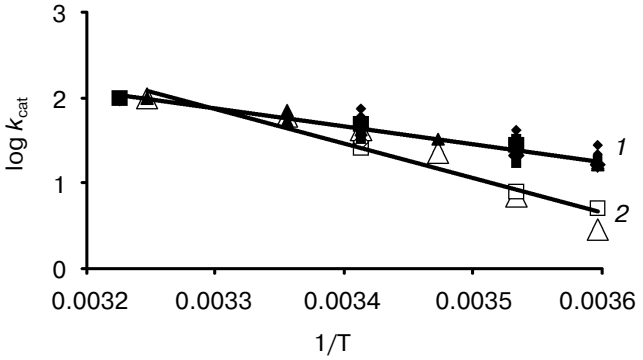

Fig. 1. Arrhenius dependence of hydrolysis of substrates by anionic trypsins: *1*) from *Paranotothenia magellanica* Forster, *Salmo gairdneri*, and rat enzymes K188F, K188Y, K188H, K188W, and Y228F; E_a = 9613 ± 393 cal/mol (49); *2*) by the bovine cationic trypsin; E_a = 18,497 ± 140 cal/mol (12). The number of experiments is given in parentheses; values for the substrate BAPNA were calculated from data of Genicot et al. [10]. N-Suc-Ala-Ala-Pro-Arg-*pNA* (dark and light diamonds); N-Suc-Ala-Ala-Pro-Lys-*pNA* (dark and light squares); BAPNA (dark and light triangles).

The temperature dependences of K_m were also calculated using normalized values (for each substrate 100% corresponded to K_m value at 37°C) because the affinity of the short substrate BAPNA for trypsin is 10-100 times

Table 1. Arrhenius activation energy (E_a, cal/mol) of hydrolysis of some substrates by anionic and cationic trypsins

Enzyme	Substrate		
	N-Suc-Ala-Ala-Pro-**Arg**-*pNA*	N-Suc-Ala-Ala-Pro-**Lys**-*pNA*	BAPNA*
Anionic trypsins			
Rat	9941 ± 636	8080 ± 562	—
P. magellanica	—	—	10 335 ± 771
Trout	—	—	9225 ± 794
K188F, K188Y, K188H, K188W, Y288F	7323 ± 640	9163 ± 152	—
Bovine cationic trypsin	12 864 ± 850	16 217 ± 710	20 490 ± 2970

* Calculated from data of Genicot et al. [10].

lower that the affinity of more effective substrates—tetrapeptide-*p*-nitroanilides. The behavior of the cationic trypsin was still more different from that of the anionic enzyme, and the temperature dependences of K_m values were significantly more complicated and depended on both the length of the substrate and on the amino acid residue, Arg or Lys, producing the amide bond to be cleaved.

The rat anionic trypsin bound the lysine-containing substrate virtually similarly over the whole temperature range (Fig. 2, curve *1*); the binding of the arginine-containing tetrapeptide amide was 40% better with decrease in temperature from 37 to 5°C (Fig. 2, curve *3*). The temperature dependences of the Michaelis constant for hydrolysis of BAPNA by fish psychrophilic trypsins (calculated from the data of Genicot et al. [10]) were rather close to curve *3*. Thus, in hydrolysis of arginine-containing substrates by natural anionic trypsins the K_m value similarly depended on temperature in the range from 5 to 37°C: it decreased about twofold with decrease in temperature, independently of the substrate length.

The temperature dependences of the k_{cat} for all mutant trypsins studied (K188F, K188Y, K188W, K188H, and Y228F) did not differ from the corresponding dependence of the wild-type enzyme, whereas the substrate binding at different temperatures was rather strongly affected by substitutions of the K188 and Y228 residues in the rat anionic trypsin. The temperature dependences of hydrolysis constants of mutant trypsins will be considered separately more in detail.

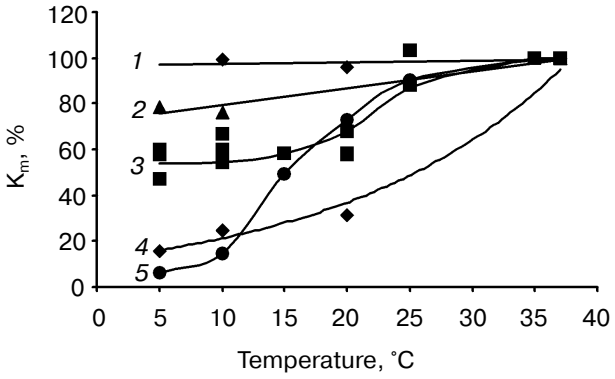

Fig. 2. Temperature dependence of the Michaelis constant (K_m) for natural anionic and cationic trypsins. Anionic trypsins: *1*) rat, substrate N-Suc-Ala-Ala-Pro-Lys-*pNA*; *3*) rat, substrate N-Suc-Ala-Ala-Pro-Arg-*pNA*; *Paranotothenia magellanica* Forster, *Salmo gairdneri* (psychrophilic), substrate BAPNA. The bovine cationic trypsin: *2*) substrate N-Suc-Ala-Ala-Pro-Arg-*pNA*; *4*) substrate N-Suc-Ala-Ala-Pro-Lys-*pNA*; *5*) substrate BAPNA.

In the case of the bovine cationic trypsin, temperature changes affected the substrate binding more markedly. The temperature dependences of K_m values inversely depended on the amino acid residue at the bond cleaved: on increase in the temperature from 5 to 37°C the total increase in the K_m value for the arginine-containing substrate was no more than 20% (Fig. 2, curve *2*) and for the lysine-containing substrate the binding decreased fivefold (Fig. 2, curve *4*).

In addition to the Arg and Lys residues in the P_1 position, these extended substrates have in the P_2 position residues interacting with the S_2-site of trypsin which are also involved in the binding to the enzyme. On temperature increase from 5 to 37°C the increase in the K_m value was the highest (16-fold) for the short substrate BAPNA, which is bound only in the S_1-site at the cost of the arginine residue (Fig. 2, curve *5*).

Absolute values of the K_m constant of natural psychrophilic trypsins at 25°C were five-ten times lower than the corresponding value for the bovine cationic trypsin with BAPNA used as the substrate [8-10] but at 5°C they became virtually the same (because of sharp improvement of BAPNA binding by the control cationic trypsin) [10]. This temperature dependence was significantly less pronounced for all anionic trypsins than for the cationic enzyme (Fig. 2, curve *3*). Thus, if mesophilic anionic trypsin was taken as the control, similar five-tenfold increase in the affinity of the "cold" trypsin for the substrate should be expected at both 5 and 25°C.

Effect of temperature on the preference of Arg/Lys by anionic and cationic trypsins. Side chains of arginine and lysine differently interact with the primary substrate-binding site of trypsin (Asp189). This is displayed kinetically by two-tenfold preference for Arg compared to Lys [14, 18], and arginine-containing substrates are characterized not only by better binding (lower values of the Michaelis constant) but also by slightly increased catalytic activity (higher values of k_{cat}) due to better orientation of the bond cleaved relative to the catalytic triad.

Obviously, the binding in the S_1-site of trypsin of the two substrates used by us, the arginine- and lysine-containing ones, should not only depend differently on temperature changes but also change differently in the case of anionic and cationic trypsins. The temperature dependences of trypsin hydrolysis found in the present work confirmed this suggestion.

The higher efficiency of hydrolysis of the arginine-containing substrate was contributed to by the better binding ($K_m^R < K_m^K$) and better catalysis ($k_{cat}^R > k_{cat}^K$). In the case of rat anionic trypsin at 37°C the contributions are approximately the same: $K_m^K/K_m^R \approx k_{cat}^R/k_{cat}^K$, but with decrease in temperature the contribution of the better binding of the arginine residue compared to that of lysine began to prevail and this increased the preference of the arginine-containing substrate due to the greater increase in the affinity for the arginine residue ($K_m^K/K_m^R : k_{cat}^R/k_{cat}^K \approx$

Table 2. Preference of the arginine-containing substrate compared to the lysine-containing one at different temperatures by the bovine cationic trypsin and rat anionic trypsin and also by mutant variants of the latter

Trypsin	37°C			20°C			10°C			5°C		
	K_m^K/K_m^R	k_{cat}^R/k_{cat}^K	Arg/Lys*	K_m^K/K_m^R	k_{cat}^R/k_{cat}^K	Arg/Lys*	K_m^K/K_m^R	k_{cat}^R/k_{cat}^K	Arg/Lys*	K_m^K/K_m^R	k_{cat}^R/k_{cat}^K	Arg/Lys*
Rat anionic	3.45	3.65	12.58	5.71	3.18	18.2	5.13	2.73	14.00	—	—	—
Bovine cationic	4.00	1.16	4.63	3.95	1.35	5.34	1.28	4.56	5.84	0.79	6.24	4.90
K188F	4.14	1.78	6.57	2.02	3.27	6.60	6.95	2.64	18.30	—	—	—
K188Y	4.00	3.42	13.70	3.36	5.64	19.00	2.34	4.85	11.40	—	—	—
K188H	2.57	2.18	5.54	2.98	2.06	6.12	6.06	1.74	10.38	1.57	3.93	6.22
Y288F	6.83	1.29	8.78	3.79	1.9	7.17	4.23	1.78	7.55	6.33	1.64	10.5

* The ratio of k_{cat}/K_m values for the arginine- and lysine-containing substrates.

2 : 1 at 10°C). The total relative efficiency of catalysis (Arg/Lys) is in the range of 13-18 (Table 2).

This dependence for bovine cationic trypsin was different: at 37°C this effect was mainly due to the better binding of the arginine-containing substrate than the better catalysis ($K_m^K/K_m^R : k_{cat}^R/k_{cat}^K \approx 3.5 : 1$). With decrease in temperature, the relative efficiency of the binding of the arginine-containing substrate began to decrease along with concurrent increase in the contribution of the better catalysis, and at 5°C the ratio $K_m^K/K_m^R : k_{cat}^R/k_{cat}^K \approx 1 : 8$. And for the first time the binding of the lysine-containing substrate was better than the binding of the arginine-containing one, but it was compensated by more than sixfold improvement of hydrolysis of the latter. As a result, the Arg/Lys ratio was close to 5 within the whole temperature range (Table 2).

Temperature dependences of catalytic parameters of mutant anionic trypsins. The comparison of known amino acid sequences of different trypsins suggested that the basic amino acid residue in position 188 located immediately close to the S_1-binding site is highly conservative. The substitution of this residue (K188 in the case of the rat anionic trypsin used by us) by site-directed mutagenesis significantly changed the enzymatic properties of the resulting mutants which, nevertheless, retained the general trypsin specificity [14-17]. The resulting anionic mutant trypsins K188F, K188Y, K188W, and K188H hydrolyzed peptide bonds produced by arginine and lysine residues but displayed some new features, such regulation of their activities by Cu^{2+} in the case of K188H [14], or a new additional specificity on hydrolysis of β-casein [15, 17], and also significant shifts of the pH optimum (K188F and K188Y [17]). Obviously, these substitutions rather strongly modified electrostatic and hydrophobic interactions near the S_1-substrate-binding site.

The substitution of lysine in position 188 by a phenylalanine residue slightly affected the k_{cat} value of both the arginine- and lysine-containing substrates over the whole temperature range (10-37°C). In the case of K188Y and K188H, the catalytic activity was somewhat decreased; introduction of a tryptophan-188 residue unfavorably influenced the catalytic properties: the mutant K188W was the least active of the mutants studied (Table 3). The substitution of tyrosine-228 residue by phenylalanine somewhat increased the catalytic activity, especially with respect to the lysine-containing substrate (twofold increase in the temperature range from 10 to 37°C) (Table 3). And the temperature dependences of k_{cat} relative values were more or less described by the general dependence for anionic trypsin (Fig. 1, Table 1).

It is more difficult to interpret the effect of substitution of the K188 and Y228 residues on the temperature dependences of K_m, but in all cases the changes were more pronounced for the arginine-containing substrate (Table 3). Maximal changes in the K_m value (50-60% increase in the temperature range from 5 to 37°C) during hydrolysis of the lysine-containing substrate by mutant trypsins were observed only in the case of Y228F. It seems that any change of the amino acid residue in position 188, as well as the temperature changes, failed to affect the binding of lysine in the S_1-site of anionic trypsins.

Based on the temperature dependences of the Michaelis constant values for the arginine-containing substrate, all mutant trypsins are divided into three groups. The temperature dependence of the K_m value for K188H and Y228F is virtually the same as for the wild-type anionic trypsin. The dependence for K188Y is inverse: the binding decreased twofold with the temperature decrease from 37 to 5°C. The temperature dependence for two other mutant trypsins, K188F and K188W, is

Table 3. Temperature dependence of kinetic constants of hydrolysis of substrates N-Suc-Ala-Ala-Pro-Arg-*pNA* and N-Suc-Ala-Ala-Pro-Lys-*pNA* (50 mM Tris-HCl buffer, 20 mM $CaCl_2$, pH 8.0) by rat anionic trypsins (the wild-type and mutant variants) and bovine cationic trypsin

t,°C	Trypsin	Suc-Ala-Ala-Pro-**Arg**-*p*-*NA*			Suc-Ala-Ala-Pro-**Lys**-*p*-*NA*		
		K_m, μM	k_{cat}, sec^{-1}	k_{cat}/K_m, $sec^{-1}\cdot\mu M^{-1}$	K_m, μM	k_{cat}, sec^{-1}	k_{cat}/K_m, $sec^{-1}\cdot\mu M^{-1}$
37	Rat	48.4 ± 0.5	174.0 ± 8.3	3.59 ± 0.10	167.0 ± 2.8	47.7 ± 5.7	0.29 ± 0.04
	K188F	46.2 ± 6.6	127.0 ± 8.9	2.43 ± 0.11	191.2 ± 12.2	71.3 ± 4.8	0.37 ± 0.03
	K188Y	53.0 ± 4.9	87.9 ± 4.2	1.66 ± 0.08	212.0 ± 7.6	25.6 ± 0.6	0.12 ± 0.02
	K188H	74.0 ± 6.6	98.6 ± 1.7	1.33 ± 0.04	190.4 ± 9.8	45.2 ± 5.4	0.24 ± 0.01
	K188W	117.8 ± 17	44.7 ± 4.9	0.38 ± 0.01	—	—	—
	Y228F	72.3 ± 0.6	135.8 ± 4.6	1.88 ± 0.03	493.9 ± 22.5	105.5 ± 15.9	0.21 ± 0.01
	Bovine	50.0 ± 0.6	110.2 ± 6.2	2.2 ± 0.04	200.1 ± 14	95.1 ± 5.1	0.48 ± 0.02
20	Rat	28 ± 1.3	75.8 ± 2.1	2.71 ± 0.08	159.9 ± 4.7	23.8 ± 2.7	0.15 ± 0.01
	K188F	36.6 ± 3.1	76.3 ± 2.7	2.09 ± 0.09	74.1 ± 8.1	23.4 ± 3.0	0.32 ± 0.01
	K188Y	80.3 ± 5.7	66.0 ± 3.7	0.82 ± 0.04	269.7 ± 13.8	11.7 ± 1.9	0.04 ± 0.01
	K188H	42.5 ± 2.9	41.6 ± 2.2	0.98 ± 0.05	126.7 ± 17	20.2 ± 1.8	0.16 ± 0.01
	K188W	—	—	—	—	—	—
	Y228F	69.6 ± 2.8	83.1 ± 7.1	1.19 ± 0.09	263.9 ± 7.4	43.7 ± 5.2	0.17 ± 0.01
	Bovine	15.8 ± 0.9	32.8 ± 4.9	2.08 ± 0.07	62.4 ± 2	24.2 ± 2.7	0.39 ± 0.02
10	Rat	32.3 ± 0.8	37.0 ± 3.5	1.15 ± 0.07	165.6 ± 4.7	13.6 ± 1.6	0.08 ± 0.01
	K188F	20.3 ± 3.5	32.8 ± 3.1	1.61 ± 0.09	141.2 ± 15.4	12.4 ± 1.9	0.08 ± 0.01
	K188Y	87.2 ± 1.5	36.2 ± 4.4	0.42 ± 0.02	204.3 ± 11.1	7.5 ± 1.1	0.04 ± 0.01
	K188H	46.2 ± 3.3	25.8 ± 1.3	0.56 ± 0.02	280.1 ± 28	15.0 ±1.3	0.06 ± 0.01
	K188W	46.1 ± 7.7	13.6 ± 2.1	0.30 ± 0.01	—	—	—
	Y228F	54.4 ± 4.6	39.1 ± 3.0	0.72 ± 0.06	230.1 ± 9.8	21.9 ± 1.9	0.095 ± 0.01
	Bovine	38.2 ± 1.1	34.5 ± 2.8	0.9 ± 0.03	49.2 ± 6.8	7.6 ± 0.9	0.15 ± 0.01
5	Rat	25.4 ± 1.1	28.2 ± 1.9	1.11 ± 0.05	—	—	—
	K188F	—	—	—	—	—	—
	K188Y	123.1 ± 17.7	25.0 ± 1.9	0.20 ± 0.01	—	—	—
	K188H	101.0 ± 13.2	33.1 ± 1.6	0.33 ± 0.02	158.4 ± 5.1	8.4 ± 1.1	0.05 ± 0.01
	K188W	—	—	—	—	—	—
	Y228F	53.0 ± 0.5	30.1 ± 2.5	0.57 ± 0.01	335.4 ± 23.2	18.3 ± 1.7	0.06 ± 0.01
	Bovine	39.4 ± 1.7	30.1 ± 1.4	0.76 ± 0.04	31.0 ± 3.3	4.8 ± 0.7	0.16 ± 0.01

noticeably different: the substrate is bound threefold more strongly at low temperatures. Absolute values of K_m for the first of these trypsins at temperatures below 20°C were also the lowest (Table 3).

In total, the temperature dependences of the relative efficiency of hydrolysis (k_{cat}/K_m) of the arginine- and lysine-containing extended substrates by mutant anionic trypsins K188H and Y228F were close to the corresponding dependences for the wild-type anionic trypsin, and in the case of K188Y the relative efficiency of hydrolysis decreased more markedly with decrease in the temperature. Relative catalytic efficiencies of hydrolysis by K188F and K188W of the Arg-containing substrate were especially high (70-80% at 10°C) (Fig. 3).

All mutants in the residue in position 188 displayed more effective hydrolysis of the arginine-containing sub-

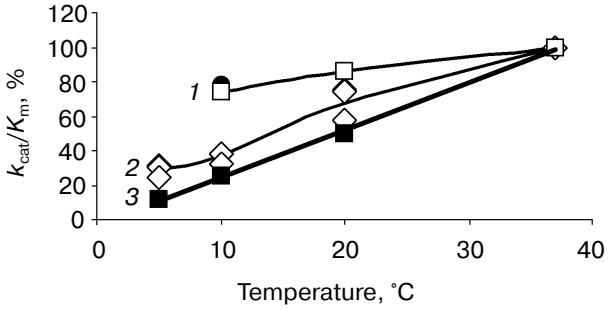

Fig. 3. Temperature dependences of relative efficiency of hydrolysis of N-Suc-Ala-Ala-Pro-Arg-*pNA* by mutant anionic trypsins. *1*) K188F, K188W; *2*) the wild-type trypsin, K188H, Y228F; *3*) K188Y.

strate than of the lysine-containing one and retained all features specific for the anionic trypsin: the Arg/Lys preference was 5-10-fold at 37°C and 8-18-fold at 5-10°C (Table 2).

The greater efficiency of hydrolysis of the arginine-containing substrate by the mutant trypsin Y228F over the whole temperature range was mainly contributed by the better binding. $K^K_m/K^R_m : k^R_{cat}/k^K_{cat} \approx 5 : 1$ at 37°C and 4 : 1 at 5°C and the ratio Arg/Lys itself (the 9-10-fold one) only weakly depended on temperature (Table 2). Thus, in this parameter Y228F was unlike either anionic or cationic trypsin.

Of two mutant trypsins for which the substrate binding was most favored by decrease in the temperature, K188W had the lowest activity in absolute figures among all mutant trypsins studied (about 10% of the wild-type trypsin activity); the efficiency of hydrolysis by K188W

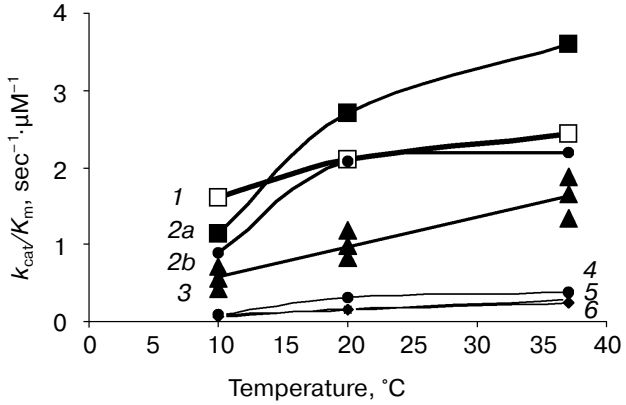

Fig. 4. Temperature dependences of the hydrolysis efficiency (k_{cat}/K_m) of the substrates N-Suc-Ala-Ala-Pro-Arg-*pNA* (by K188F (*1*), by the rat wild-type trypsin (*2a*), by the bovine cationic trypsin (*2b*), by K188Y, K188H, Y228F (*3*)) and N-Suc-Ala-Ala-Pro-Lys-*pNA* (by K188F (*4*), by the rat wild-type trypsin (*5*), by K188Y (*6*)).

was low because of the low k_{cat} value even at 37°C. The introduction of a bulky tryptophan residue into position 188 seemed to affect the geometry of the active site of the enzyme in spite of the high affinity for the substrate, especially at 10°C.

Introduction of phenylalanine instead of lysine into position 188 had virtually no effect on the catalytic constant over the temperature range studied and concurrently markedly increased the binding of the arginine residue in the substrate-binding pocket. As a result, K188F had the highest catalytic efficiency at low temperatures among the mutant and wild-type trypsins studied (Fig. 4). The improvement of binding of the Lys residue induced by decrease in the temperature was less noticeable; however, over the temperature range from 10 to 37°C K188F also had the highest catalytic activity (Fig. 4).

It is also worth attention that the pH optimum of K188F was shifted to alkalinity [17], although direct correlation between the psychrophilicity and pH optimum at 9-10 has not been proved for natural "cold" trypsins. Certainly, the 1.5-fold increase in the catalytic efficiency of the rat anionic trypsin at low temperatures induced by substitution of the lysine residue in position 188 by phenylalanine was much less than the corresponding effects of natural psychrophilic trypsins (10-20-fold). Nevertheless, because at present we are far from understanding molecular mechanisms of adaptation for low temperatures, the model chosen of the effect of the adjacent amino acid residues on the S_1-site of trypsin seems promising, and the hypothesis of Smalas et al. [5] which speculates about these phenomena seems reasonable. The finding of the present work can be generalized as follows.

1. Anionic and cationic forms of trypsin have differences in the organization of the S_1-binding site.

2. Secondary interactions of the peptide chain of the substrate compensate significant changes in the binding of the P_1-residue of the substrate in the primary binding pocket that are induced by temperature changes (data only for cationic trypsin).

3. The different binding of lysine and arginine residues in the S_1 site is also displayed by opposite effects of temperature changes on the comparative efficiency of hydrolysis of the arginine- and lysine-containing substrates by natural anionic and cationic trypsins.

4. Effects of any factor on the binding of substrates (the K_m value) and on the catalytic activity (k_{cat}) for anionic trypsins should be compared only to the corresponding data for the natural anionic enzyme.

5. The efficiency of catalysis by natural psychrophilic trypsins was 10-20 times higher at both 25 and 5°C than by mesophilic trypsin.

6. Introduction of a phenylalanine residue into position 188 of the rat anionic trypsin increased 1.5-fold the catalytic efficiency of the corresponding mutant trypsin K188F at low temperatures as compared to the wild-type enzyme.

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