ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 8, pp. 824-833. © Pleiades Publishing, Ltd., 2009.
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Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-286, March 1, 2009.

Effect of Reactive Center Loop Structure of Antichymotrypsin on Inhibition of Duodenase Activity

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Received September 8, 2008
Revision received November 17, 2008

Abstract—Interaction between duodenase (a granase family member) from bovine duodenal mucosa and recombinant antichymotrypsin (rACT) and its P1 variants has been studied. Association rate constants (k_a) were 11, 6.8, and 17 $mM^{-1}\cdot sec^{-1}$ for rACT, ACT L358M, and ACT L358R, respectively. Natural antitrypsin (AT) compared to ACT was a 20 times more effective duodenase inhibitor (in terms of k_a). Duodenase interacted with P1 variants of ACT via a suicide mechanism with stoichiometry of the process SI = 1.2. The nature of the P1 residue of the inhibitor did not influence the interaction if other residues did not meet conformational requirements of the duodenase substrate-binding pocket. Also, interaction of duodenase with ACT variants containing residues from AT reaction center loop (rACT P2-P3′, rACT P3-P4′, rACT P4-P3′, and rACT P6-P4′) was studied. The inhibition type ($[E]_0 = 1\cdot10^{-7}$ M, 25°C) was revealed to be reversible-like, and efficacy of inhibition decreased with increase in the substituted part of the reactive center loop. Constants of inhibition (K_i) were measured. Efficacy of interaction between the enzyme (duodenase) and inhibitor depends on topochemical correspondence between a substrate-binding pocket of the enzyme and substrate structure.

DOI: 10.1134/S0006297909080021

Key words: duodenase, serpins, conformational specificity, granase

Duodenase (EC 3.4.21), a dominant serine protease from bovine duodenal mucosa, is a secretion product of duodenal glands [1]. The physiological role of duodenase is supposed to be associated with activation of the digestive protease cascade [2]. Alternative localization of duodenase in mast cells also implies involvement of the enzyme in processes associated with defense reactions [3]. Structurally, duodenase is a member of the granase family, a specific mammalian granular protease group comprising granzymes, chymases, and cathepsin G [4]. Members of this group have lost an ancient Cys191–Cys220 disulfide bond in their active centers (numeration following chymotrypsinogen), which is strongly conservative in classical serine proteases (pancreatic proteases, blood clotting and fibrinolysis factors, and some others), thus acquiring some peculiarities of catalytic features [4].

Broadened primary specificity (trypsin- and chymotrypsin-like) of duodenase is combined with expressed selectivity in regards to recognition and hydrolysis of protein substrates and inhibitors [1, 5]. Determination of factors naturally regulating duodenase seems important because of the putative physiological importance of duodenase. According with the results of earlier studies, endogenous serpins, which are immanently present in small intestine mucosa, are possible regulators of duodenase activity *in vivo* [6]. The presence of a labile reaction center loop (RCL) exposing the bond hydrolyzed by the target protease is a feature of serpins. Substantial conformational changes occur in the inhibitor cleft by the protease followed by the formation of a quite stable covalent complex with the protease [7]. Duodenase can be effectively inhibited *in vitro* by α1-antitrypsin (AT) and, to a lesser extent, by antichymotrypsin (ACT) from human blood serum. The interaction between the enzyme and these inhibitors occurs via the classical suicide mecha-

Abbreviations: (r)ACT, (recombinant) antichymotrypsin; AT, α1-antitrypsin; BBI, soybean Bowman–Birk inhibitor; BTEE, N-benzoyl-L-tyrosine ethyl ester; CT, chymotrypsin; RCL, reaction center loop; SI, stoichiometry of inhibition.

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nism with the formation of stable enzyme–inhibitor complex [6]. The paradox is that duodenase hydrolyses the bond formed by Met P1 residue in the AT reaction loop, whereas the hydrolysis of the Met–X bond is virtually absent in the studied peptide and protein substrates of duodenase. The protease-hydrolyzed bond in ACT RCL is formed by Leu, which is rather acceptable as the P1 residue in substrate hydrolysis by duodenase, but ACT is 20 times less active in regards to this enzyme.

The interaction of duodenase with mutant ACT forms containing an altered P1 residue of hydrolyzed bond, as well as with chimeric ACT variants with substituted RCL fragment by AT fragment of the same length, was studied with the aim to determine the role of structural features of serpin RCL in duodenase inhibition and to characterize the factors determining affinity of duodenase to a particular substrate or inhibitor.

MATERIALS AND METHODS

N-Benzoyl-L-tyrosine ethyl ester (BTEE), SucAlaAlaProPhe-pNA, phenylmethylsulfonyl fluoride (PMSF), N-*trans*-cinnamoyl imidazole, and soybean Bowman–Birk inhibitor (BBI) were purchased from Sigma (USA); dValLeuLys-pNa (S 2251) was from Kabi (Sweden); chymotrypsin (CT) (54% active centers) was from Fluka (Switzerland); Tris, dimethylsulfoxide (DMSO), acrylamide, and N,N′-methylene-bis-acrylamide were from ICN (USA). N,N,N′,N′-Tetramethylethylenediamine (Temed) was from Merck (Germany); ammonium persulfate was from Reanal (Hungary); and Coomassie blue G-250 was from Fluka (Switzerland).

ACT mutants were kindly provided by Dr. Michael Plotnick (University of Pennsylvania, USA).

Purification of duodenase. Duodenase was purified according to the method of Zamolodchikova et al. [10]. The number of active centers in purified duodenase was determined by titration of the enzyme with classical soybean inhibitor from the Bowman–Birk family (BBI) as described earlier [11]. Here and further, the protease activity was measured using a UV-265 FW spectrophotometer (Shimadzu, Japan) at 25°C.

Measurement of active mutant concentrations. Concentration of inhibitors was determined by titration of chymotrypsin (CT) with known concentration of active centers determined by the titration with N-*trans*-cinnamoyl imidazole. Ten microliters of CT solution in 1 mM HCl (0.1 mg/ml, 54% active centers) and different recombinant ACT (rACT) dissolved at 100-fold concentration in working buffer (0-30 μl of rACT P2-P3′, 0-100 μl of rACT P3-P4′, 0-50 μl of rACT P4-P3′, or 0-70 μl of rACT-P6-P4′) were added into buffer solution of 0.05 M Tris-HCl, pH 8.0, taken to the final volume of 1 ml and incubated for 15 min. Then 80 μl of BTEE solution in methanol (0.5 mg/ml) was added, and the change in optical density was registered at 256 nm. Then the plot of residual CT activity against the volume of the inhibitor added was linearly extrapolated to determine the inhibitor volume necessary for complete inhibition, and the active inhibitor concentration was calculated.

Determination of dependence of duodenase activity on [inhibitor]/[enzyme] ratio. A) *Point ACT mutants (rACT WT*, *rACT L358R*, *rACT L358M)*. Ten microliters of 17 μM duodenase solution in 0.01 M phosphate-citrate buffer, pH 4.5, was added to 480 μl of 0.034-0.34 μM point mutant solution in 0.05 M Tris-HCl buffer (pH 8.0) and incubated for 20 min. Then 20 μl of 10 mM SucAlaAlaProPhe-pNA substrate was added, and the change in optical density was registered at 410 nm. The plot of residual duodenase activity against the initial inhibitor/duodenase concentration ratio was linearly extrapolated to the point of intersection with the abscissa to determine the inhibitor/enzyme ratio necessary for complete inhibition.

B) *Chimeric rACT forms (rACT P6-P4′*, *rACT P3-P4′*, *rACT P4-P3′*, *rACT P2-P3′)*. Ten microliters of duodenase solution (7.2 μM) in 0.01 M phosphate-citrate buffer, pH 4.5, was added to 670 μl 0.05 M Tris-HCl buffer, pH 8.0, containing 0-1.1 μM rACT P2-P3′, 0-5 μM rACT P3-P4′, 0-2 μM rACT P4-P3′, or 0-6.2 μM rACT P6-P4′ and incubated for 20 min. Then 20 μl of dValLeuLys-pNA (5 mM) was added, and the change in optical density at 410 nm was registered. Equation (1) was used to determine the inhibition constant $K_{i(app)}$ [12]:

$$a = 1 - \frac{\left([E]_0 + [I]_0 + K_{i(app)}\right) - \left\{\left([E]_0 + [I]_0 + K_{i(app)}\right)^2 - 4[E]_0[I]_0\right\}^{1/2}}{2[E]_0}. \quad (1)$$

The true K_i value was calculated according to Eq. (2):

$$K_{i(app)} = K_i\,(1 + [S]_o/K_m). \quad (2)$$

In these equations, a is the relative enzymatic activity, $[E]_0$ is the initial enzyme concentration, $[I]_0$ is the initial inhibitor concentration, $K_{i(app)}$ is the apparent inhibition constant, K_i is the true inhibition constant, $[S]_0$ is substrate concentration, K_m = 0.4 mM is the Michaelis constant for dValLeuLys-pNA substrate.

Measurement of the inhibition rate constants (k_a). Curves of product accumulation were obtained under conditions of pseudo first-order reaction mechanism in the presence of substrate according to Petersen and Clemmensen [13]. The reaction mixture contained 0.4 mM dValLeuLys-pNA substrate, 34-525 nM of duodenase, and point ACT mutant (with the exception of rACT) at concentration sufficient for supporting the ratio $[I]_0/[E]_0$ = 10. The concentration of *p*-nitroaniline formed was registered at 410 nm, and the values obtained were differentiated to obtain the dependence of the reac-

tion rate on time. The apparent first order rate constant (k_{obs}) was calculated using non-linear regression according to the Eq. (3). Association rate constant (k_a) was determined according to the Eq. (4), where [I] is inhibitor concentration, [S] is substrate concentration, and K_m is the Michaelis constant for dValLeuLys-pNA:

$$V = V_0 e^{-k_{obs}t}, \quad (3)$$

$$k_{obs} = k_a[I]\left(1 + \frac{S}{K_m}\right)^{-1}. \quad (4)$$

The rate constant of association of duodenase with rACT was measured by an analogous method, but at constant initial enzyme concentration (34 nM) and varied initial rACT concentration from 0.34 to 3.4 μM.

Study of interaction between duodenase and ACT mutants by SDS-PAGE. Samples were prepared as follows. A) Three microliters of duodenase solution (1.4 μg) in 0.01 M acetate buffer, pH 4.5, and 5 μl of point ACT mutant solution (2.3 μg) were added to 8 μl of 0.05 M Tris-HCl buffer, pH 8.0. The mixture was incubated for 5-120 min at 25°C, and then the reaction was terminated by addition of 2 μl of 2 mM PMSF solution in isopropanol followed by incubation for 15 min. Then 4 μl of five-fold sample buffer was added. The samples were incubated at 80°C for 10 min and centrifuged prior to the loading onto the gel. The same amounts of duodenase and mutants prepared by the method described earlier were used as controls.

B) Ten microliters of duodenase (1.8 μg) in 0.01 M acetate buffer, pH 4.5, and 2 μl of the inhibitor solution in the initial buffer (containing 12 μg ACT P2-P3′, 6.7 μg ACT P3-P4′, 6.7 μg ACT P6-P4′, or 10.6 μg ACT P4-P3′) were added to 150 μl 0.05 M Tris-HCl buffer, pH 8.0. The mixture was incubated for 15 min at 25°C or 2 h at 37°C, then 21 μl of concentrated trichloroacetic acid (TCA) was added followed by incubation for 10 min and centrifugation for 15 min at 12,000 rpm. Then the solution was decanted, and the pellet was washed twice with 10% aqueous TCA solution and then with acetone with subsequent centrifugation under the same conditions. The pellet was dissolved in 15 μl of the sample buffer, and 5 μl of the concentrated Tris solution was added for removal of the residual TCA. Thereafter the total volume of the sample was loaded into the gel well. The inhibitor samples taken in amounts used in the reaction, as well as duodenase (1.8 μg) in 150 μl of 0.05 M Tris-HCl buffer treated in the same way were used as controls.

SDS-PAGE following Laemmli [14] in 12% (see further Fig. 3a) and 8% (Fig. 3, b and c) gels was carried out using a Mini Protean II cell (Bio-Rad, USA). The electrophoresis was carried out at 20°C for 40 min at 200 mV. The gels were stained for 15 min with solution containing 0.15% Coomassie brilliant blue R-250, 30% methanol, and 10% acetic acid. Then the gels were washed overnight in solution containing 10% methanol and 10% acetic acid.

Study on duodenase interaction with ACT mutants using mass-spectrometry. Fifteen microliters of inhibitor solution (containing either 8.3 μg rACT WT, 7.6 μg rACT L358R, 14.5 μg rACT L358M, 12.4 μg rACT L358W, 35.9 μg rACT P2-P3′, 20 μg rACT P3-P4′, 20 μg rACT P6-P4′, or 31.7 μg rACT P4-P3′) and 50 μl of 0.05 M Tris-HCl buffer were added to 15 μl of duodenase solution (2.7 μg) in 0.01 M acetate buffer, pH 4.5. The mixture was incubated for 2 h at 37°C and then treated with TCA as in the previous experiment. The pellet was used for mass spectroscopy. The same amounts of the enzyme and mutants at the same concentrations treated with TCA were used as controls.

RESULTS

Inhibition of duodenase by rACT and its mutant forms with substituted P1 residue of the reactive center loop (stoichiometry and association rate constants). Figure 1 shows results of duodenase titration with recombinant ACT (rACT WT) and its mutant forms rACT L358R and rACT L358M. One of the characteristic features of the interaction between protease and serpin is the stoichiometry of inhibition (SI) whose empirical value is determined as molar amount of the inhibitor necessary for inactivation of one mole of enzyme [7]. Stoichiometry value in the case of serpins is determined from the rate constants of dissociation and stabilization of the acyl-enzyme, but not from the amount of active centers of the inhibitor, as for canonical inhibitors, and represents a unique value for each serpin–protease pair. The value of SI with duode-

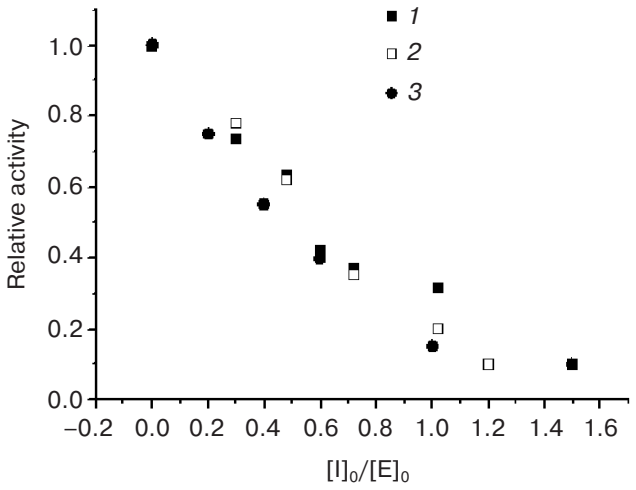


Fig. 1. Titration of duodenase with ACT-P1 variants: *1*) rACT L358M; *2*) rACT WT; *3*) rACT L358R. $[E]_0$ = 0.17 μM, incubation time 20 min. The substrate was 0.4 mM dValLeuLys-pNa. The reaction buffer was 0.05 M Tris-HCl, pH 8.0.

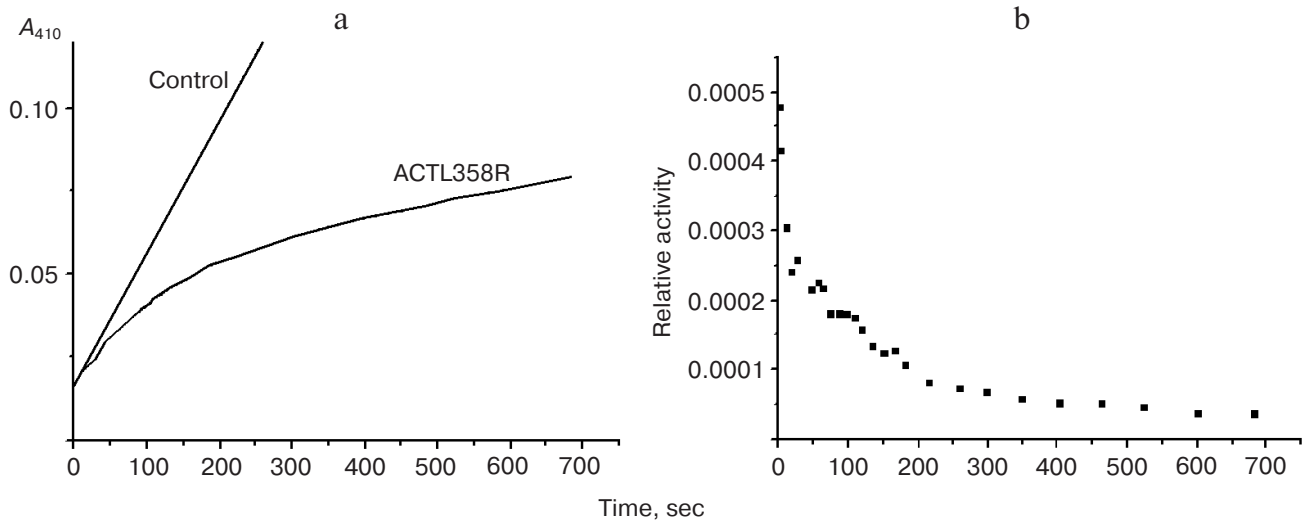


Fig. 2. Product accumulation (a) and duodenase inactivation rate (b) in the presence of rACT L358R. The control does not contain inhibitor. Substrate, 0.4 mM dValLeuLys-pNa. $[I]_0/[E]_0 = 10$. Duodenase concentration 34 nM. The reaction buffer was 0.05 M Tris-HCl, pH 8.0.

nase determined in this experiment is equal for all three inhibitors, and its value is 1.2, which coincides with SI value previously determined for natural ACT [6]. The kinetics of duodenase inhibition by these ACT variants were analyzed under the conditions of pseudo first order reaction (at $[I]_0 = 10[E]_0$) in the presence of substrate. Mathematical processing of the kinetic data presented as a function of decrease in the hydrolysis reaction rate with time (Fig. 2, a and b) in accordance with the Eqs. (3) and (4) gave the rate constants (k_a) for association of duodenase with recombinant ACT (wild type and mutant forms) and natural ACT (Table 1). The values of k_a are given for comparison, characterizing the interaction of the same inhibitors with cathepsin G, the nearest structural analog of duodenase, as well as with CT and trypsin. The substitution of natural Leu358 P1 residue in ACT by Met typical for P1 position of the AT reacting loop resulted in insignificant decrease in k_a value in comparison with rACT WT, whereas the introduction of positively charged Arg residue in P1 position produced a more effective inhibitor. Thus, the arginine residue is preferred for duodenase as P1 residue in the reacting loop of the inhibitor compared to uncharged residues. This corresponds to primary specificity of duodenase hydrolyzing Arg-containing substrates more effectively than substrates with a Leu residue in P1 position [3]. A methionine residue was not found in P1 position in the studied duodenase substrates. Only a single example has been noted when duodenase hydrolyzed a peptide bond formed by the carboxyl group of a Met residue [1].

SDS-PAGE of reaction products of duodenase with rACT, rACT-L358M, and rACT-L358R. The SI value characterizing the interaction of duodenase with rACT and its mutant forms containing substitutions in P1 position is near one, which is an evidence for complex formation in interaction of duodenase with these inhibitors.

Table 1. Association constant values of duodenase, cathepsin G, chymotrypsin, and trypsin with natural and recombinant (wild-type and mutant) serpin forms. Asterisks indicate the data for the natural inhibitor forms

Enzyme \ Inhibitor	k_a, $M^{-1}\cdot sec^{-1}$				
	AT	ACT	rACT-L358M	rACT-L358R	rAT-M358R
Duodenase	$1.9 \cdot 10^5$ [15]	$1.1 \cdot 10^4$ (*) $9.5 \cdot 10^3$	$6.8 \cdot 10^3$	$1.7 \cdot 10^4$	no data
Cathepsin G	$1.6 \cdot 10^4$	$8.1 \cdot 10^5$	$1.0 \cdot 10^6$	$5.0 \cdot 10^4$	$2.9 \cdot 10^3$ [16]
Chymotrypsin	$3.6 \cdot 10^6$	$5.5 \cdot 10^5$	$4.6 \cdot 10^5$	$4.6 \cdot 10^4$	$3.5 \cdot 10^5$ [16]
Trypsin	$1.3 \cdot 10^5$ (*) [17]	$<5 \cdot 10^2$ [8]	$<10^2$ [8]	$4.1 \cdot 10^5$ [18]	$4.2 \cdot 10^5$ [18]

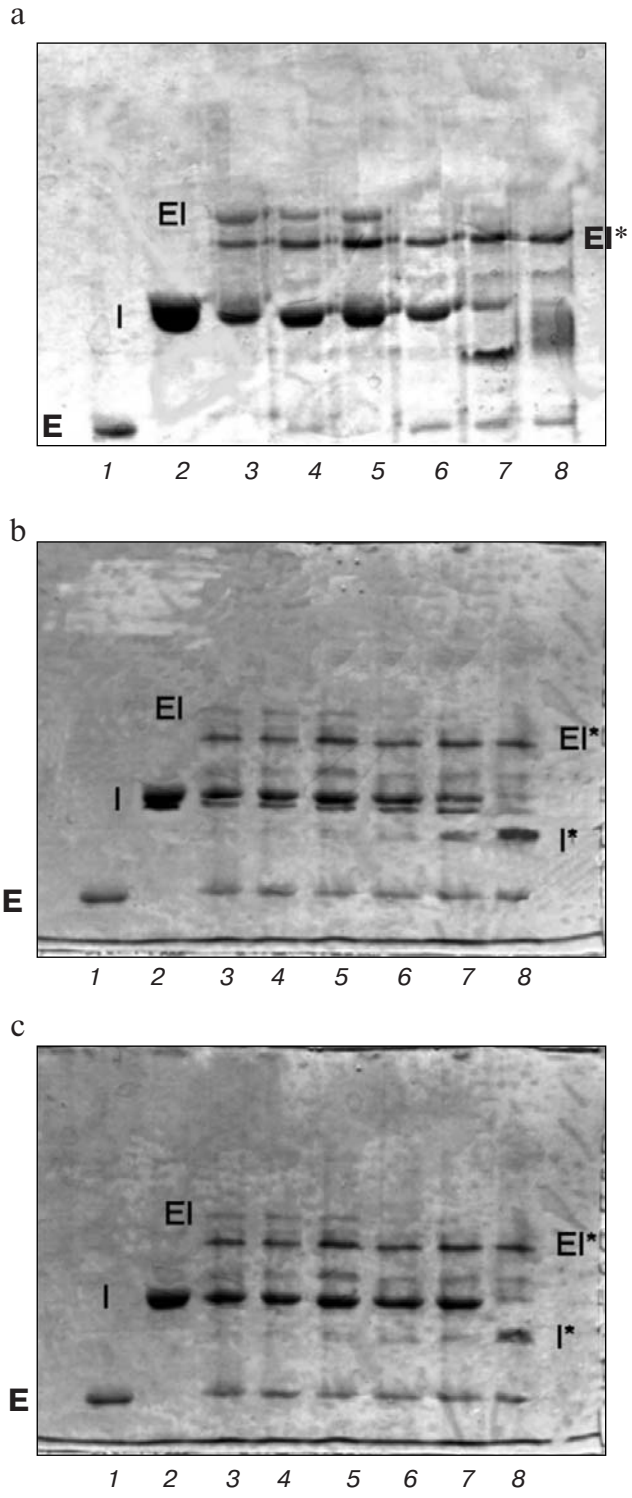
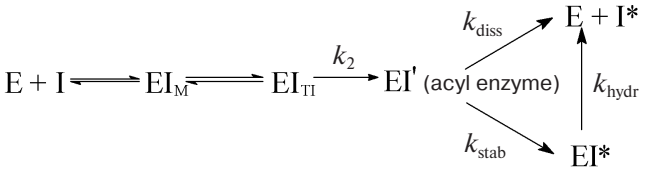


Fig. 3. SDS-PAGE analysis of products formed in the reaction of duodenase (1.4 μg) with ACT-L358M (2.3 μg) (a), ACT-L358R (2.3 μg) (b), and rACT WT (2.3 μg) (c). Lanes: *1*) duodenase; *2*) inhibitor; *3*) incubation time 5 min; *4*) 10 min; *5*) 20 min; *6*) 40 min; *7*) 60 min; *8*) 120 min. E, duodenase; I, inhibitor; I*, the cleaved inhibitor in the active center area; EI and EI*, covalent enzyme–inhibitor complex containing undegraded (I) or degraded (I*) inhibitor.



Suicide mechanism of proteinase inhibition by serpins. EI_M, enzyme–inhibitor Michaelis complex; EI_{TI}, tetrahedral intermediate; EI′, acyl enzyme; EI*, covalent complex in which the inhibitor has been cleaved (insertion of the reaction center loop into the β-sheet A occurs); I*, cleaved inhibitor. k_2, rate constant for conversion of the tetrahedral complex into the acyl enzyme; k_{diss}, rate constant for dissociation of acyl enzyme to free enzyme and cleaved inhibitor; k_{stab}, rate constant of "stabilization" of acyl enzyme (conversion into a covalent complex with conformationally altered inhibitor); k_{hydr}, rate constant for dissociation of the covalent complex with the conformationally altered inhibitor to free enzyme and cleaved inhibitor

Figure 3 shows the results of electrophoretic analysis of products of duodenase interaction with recombinant rACT WT and the rACT L358M and rACT L358R variants for various incubation durations. In all these cases, a high molecular weight band appears corresponding to a duodenase–serpin complex, which remains stable for 20 min. Under more prolonged incubation the high molecular weight band disappears, and more electrophoretically mobile bands appear corresponding to the hydrolysis products and cleaved inhibitor. Note that in the case of ACT L358M almost all of the enzyme is bound in the complex with the inhibitor (Fig. 3a), whereas almost half of the duodenase pool remains free in the reaction with ACT L358R and rACT WT, apparently due to the lower stability of the corresponding covalent complexes.

Thus, the results confirm the interaction of duodenase with rACT and its mutant forms via a classical suicide mechanism (Scheme) [7].

Interaction of duodenase with chimeric rACT forms containing several substitutions of residues in the RCL near the P1-P1′ bond by corresponding AT residues. As we pointed out earlier, natural AT and ACT inhibit duodenase, whereas AT is 20 times more effective than rACT (Table 1). The study on the interaction of duodenase with rACT chimeric structures seemed reasonable to elucidate the structural factors influencing the interaction of duodenase with serpins. Table 2 presents the sequences of the reacting loop sites near the P1–P1′ bond of the studied inhibitors. The rACT mutant forms with prolonged substituted site (from 5 to 9 residues) in the RCL inhibitor by the corresponding AT residues were produced in works on the study of the role of P6-P3′ in formation and decomposition of the ACT complex with leukocyte elastase [9]. All the mutants effectively inhibited CT and could variously inhibit elastase, which represents a natural target of

antitrypsin, whereas the natural ACT is completely inactive against this enzyme.

Figure 4 presents data on titration of duodenase (a) and CT (b) with chimeric rACT variants. The titration curves of duodenase with chimeric rACT variants basically differ from the results of the titration of CT with these mutants and from curves of duodenase titration with natural ACT and AT [6], as well as with rACT P1 mutants (Fig. 1). None of the studied chimeric variants inhibited duodenase at equimolar inhibitor/enzyme ratio (Fig. 4a). Notable inhibition was only achieved at four-fold excess of the inhibitor (rACT P2-P3′ and rACT P4-P3′). In all cases, complete inhibition was not achieved up to 50-fold excess of inhibitor. The efficacy of duodenase inhibition decreased with the increase in length of the altered RCL site in the mutant inhibitor. Mutations involving P4′ clearly negatively influence the inhibitory activity of the serpin.

The titration curve of duodenase with chimeric rACT is hyperbolic, which is more typical for reversible inhibition. The results of chymotrypsin titration (at enzyme concentration five times lower than that of duodenase) presented as a plot yields a straight line reflecting decrease in enzymatic activity with increase in inhibitor/enzyme ratio (Fig. 4b). Complete CT inhibition is achieved at equimolar $[inhibitor]_0/[enzyme]_0$ ratio for all tested chimeric rACT forms [9].

The analysis of products of duodenase reaction with chimeric rACT variants under conditions similar to those for the enzyme titration (25°C, a 15-min incubation, $[E]_0$ = 0.38 μM, $[I]_0/[E]_0$ = 2.5-4.4) by electrophoresis did not reveal bands corresponding to cleaved inhibitor.

Table 2. Reaction loop structures of ACT, AT, and chimeric rACT. Residues forming the bond being cleaved are given in bold

	P15 P6 **P1 P1'** P4'
ACT	G T E A S A A T A V K I T L **L S** A L V E
rACT P2-P3'	P **M S** I P
rACT P3-P4'	I P **M S** I P P
rACT P4-P3'	A I P **M S** I P
rACT P6-P4'	L E A I P **M S** I P P
AT	G T E A A G A M F L E A I P **M S** I P P E

Weak bands corresponding to trace amounts of complex between duodenase and either rACT P3-P4′ or rACT P4-P3′ were detected (Fig. 5a). It is obvious that the rate of duodenase reaction with chimeric rACT forms is substantially lower than the rate of the interaction between the enzyme and wild-type ACT and AT. Covalent complex with chimeric serpin (acyl enzyme) is virtually not formed under the titration conditions (25°C, $[E]_0$ = 0.1 μM), while the interaction between duodenase and these chimeric rACT forms does not follow the substrate mechanism (products of the cleavage of the inhibitor are absent). Apparently, the observed inhibition of duodenase (to 80%) under the conditions of titration with chimeric

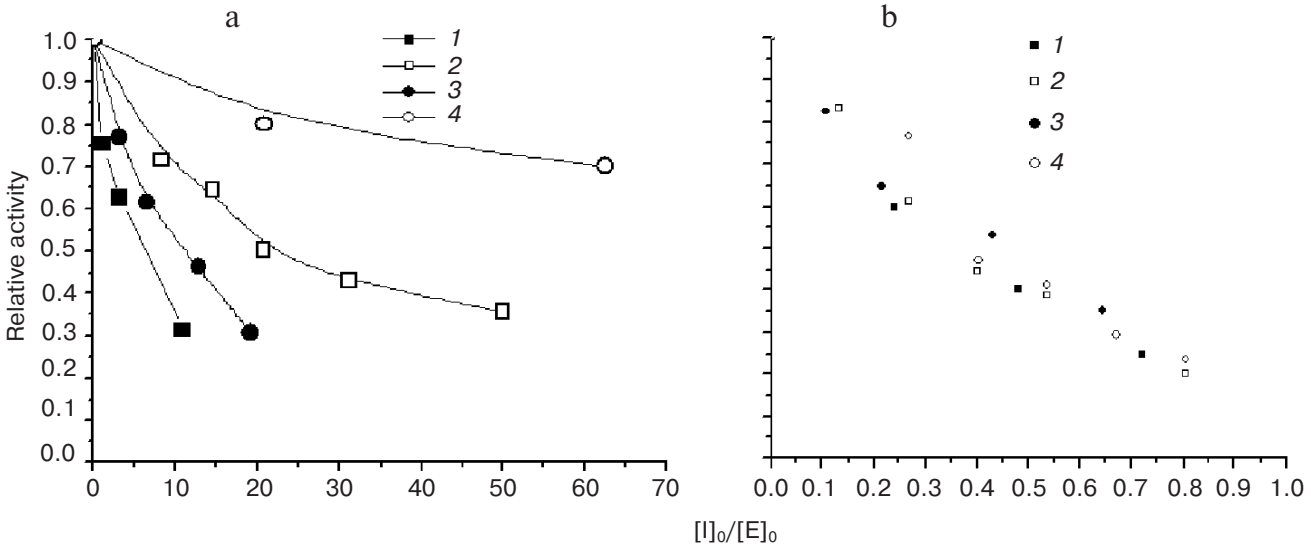


Fig. 4. a) Titration of duodenase by chimeric rACT variants: *1*) ACT P2-P3′; *2*) ACT P3-P4′; *3*) ACT P4-P3′; *4*) ACT P6-P4′. $[E]_0$ = 0.1 μM; duration of incubation was 20 min; 0.4 mM dValLeuLys-pNa was used as a substrate. Reaction buffer was 0.05 M Tris-HCl, pH 8.0. b) Chymotrypsin titration with ACT-AT variants: *1*) ACT P2-P3′; *2*) rACT P3-P4′; *3*) rACT P4-P3′; *4*) rACT P6-P4′. $[E]_0$ = 21 nM; duration of incubation was 15 min; BTEE was used as a substrate.

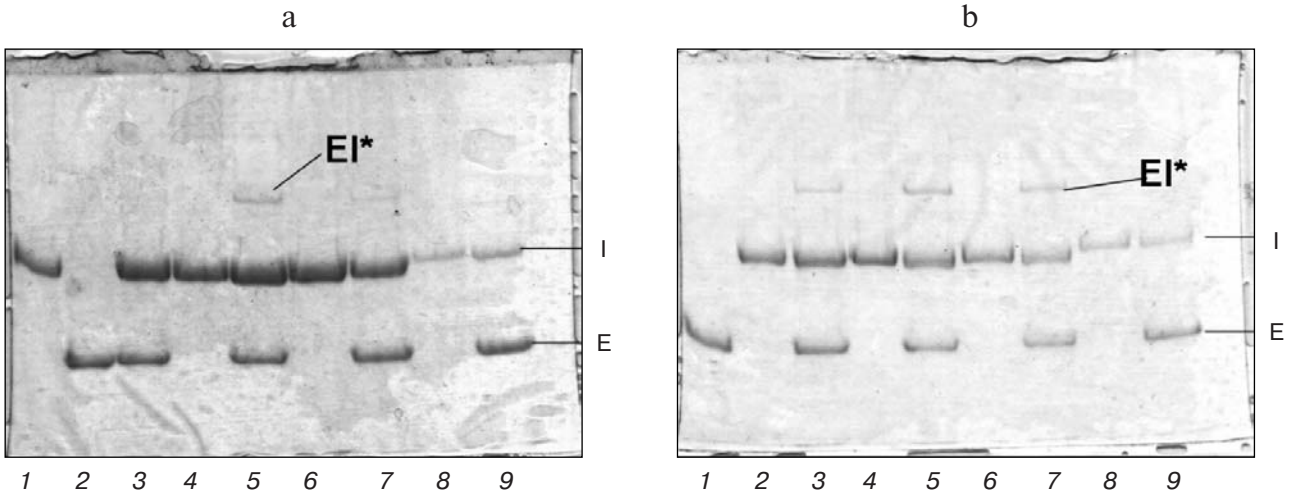


Fig. 5. a) Interaction of duodenase and chimeric rACT variants at 25°C, incubation for 15 min (SDS-PAGE in 12% gel under non-reducing conditions). Lanes: *1*) rACT P2-P3′ (12 μg); *2*) duodenase (1.8 μg); *3*) duodenase–rACT P2-P3′ reaction; *4*) rACT P3-P4′ (6.7 μg); *5*) duodenase–rACT P3-P4′ reaction; *6*) rACT P4-P3′ (6.7 μg); *7*) duodenase–rACT P4-P3′ reaction; *8*) rACT P6-P4′ (10.6 μg); *9*) duodenase–rACT P6-P4′ reaction. b) The same at 37°C, incubation for 120 min. Lanes: *1*) duodenase (1.8 μg); *2*) ACT P2-P3′ (12 μg); *3*) duodenase–rACT P2-P3′ reaction; *4*) rACT P3-P4′ (6.7 μg); *5*) duodenase–rACT P3-P4′ reaction; *6*) rACT P4-P3′ (6.7 μg); *7*) duodenase–rACT P4-P3′ reaction; *8*) rACT P6-P4′ (10.6 μg); *9*) duodenase–rACT P6-P4′ reaction. E, duodenase; I, inhibitor; EI*, covalent duodenase–inhibitor complex.

rACT variants under substantial excess of the inhibitor (Fig. 4a) occurs as a result of noncovalent interaction with the inhibitor via a reversible mechanism, as in the case of canonical inhibitors [19]. Thus, equilibrium constants of inhibition (K_i) can be calculated from the data obtained from experiments on titration of duodenase with chimeric rACT forms.

The K_i values for the interaction between duodenase and chimeric rACT variants are presented in Table 3. The inhibition constants for duodenase inhibition (by rACT P2-P3′ and rACT P4-P3′) are one order higher than those for the interaction between the enzyme and AT [15], five times higher than K_i for the inhibition of cathepsin G by ACT, and are similar to the K_i values calculated for the inhibition of elastase from human neutrophils by AT [20]. The interaction of duodenase with other chimeric rACT variants is characterized by K_i values six times higher (rACT P3-P4′) or two orders higher (rACT P6-P4′) than the respective constants for rACT P2-P3′ and rACT P4-P3′. Obviously, introduction of a Pro residue into the RCL of ACT changes its conformational properties, which in turn is reflected on the stage of recognition and binding of the loop as a substrate by duodenase and results in decrease in the affinity of the enzyme to such an inhibitor.

Table 3. Constants (K_i) of duodenase, cathepsin G, and leukocyte elastase inhibition by ACT, AT, and chimeric rACT structures

Inhibitor	K_i, M		
	duodenase	cathepsin G	elastase
AT	$1.3 \cdot 10^{-8}$ [15]	$8.1 \cdot 10^{-7}$ [20]	$(3.8\text{-}5.2) \cdot 10^{-7}$ [20]
rACT P2–P3'	$3.3 \cdot 10^{-7}$		
rACT P4–P3'	$4.3 \cdot 10^{-7}$		
rACT P3–P4'	$1.9 \cdot 10^{-6}$		
rACT P6–P4'	$3.3 \cdot 10^{-5}$		
ACT		$6.2 \cdot 10^{-8}$ [20]	

We demonstrated complex formation between serpin and duodenase for all chimeric rACT forms with the exception of rACT P6-P4′ under molar excess of the inhibitor and elevation of temperature up to 37°C and increase of the incubation time of duodenase with mutant inhibitors to 2 h (Fig. 5b). The bands corresponding to the inhibitor–protease complex are weak for all rACT mutant forms.

Thus, in accordance with the electrophoretic data, the mechanism of duodenase interaction with chimeric rACT structures carrying an insertion of AT homologous sequence in the P1–P1′ site of the reacting loop remains essentially the same as for natural serpins, that is, corresponds to the Scheme. However, the rates of interaction between duodenase and such mutants are substantially lower than in the case of natural AT and ACT.

Mass-spectrometry of products of duodenase reaction with ACT variants. Mass-spectrometric analysis of products of the reaction of duodenase with ACT variants and its wild type suggests for hydrolysis of P1–P1′ bond

(residues 358-359) in RCL of all ACT variants. It is the bond whose hydrolysis is typical for interaction of this serpin with target protease (Table 2). Thus, the site of primary recognition of the RCL sequence of ACT mutants by duodenase was not shifted due to the alteration in its structure.

DISCUSSION

The present study aimed for detailed investigation of enzymatic properties of duodenase and elucidation of the causes underlying the apparent anomaly of the enzyme specificity to protein substrates and inhibitors.

The 3-D structure of human ACT, for which X-ray structural data are absent, has been simulated [21] by the method of homologous molecular modeling using the known X-ray data for human AT [22] and murine ACT (accession code in PDB database is 1YXA). The molecular dynamic method demonstrated less stable conformational state of ACT RCL as compared with AT due to the absence of proline residues in P2, P3′, and P4′ positions, which is typical for AT. However, there are stabilizing interactions of charged residues of ACT RCL and the molecular core. Thus, the RCL of natural serpins is a rather labile structure, whose conformational state is determined by amino acid sequence of the loop and its noncovalent interactions with the globular part of the molecule, individually for each serpin. Mechanical transfer of a prolonged site of the loop from a distinct class of serpin to another structure can lead to global disturbances of natural conformational properties of such chimeric molecules due to the fault of noncovalent interactions between RCL and core because of the lack of complementarity in intramolecular interactions. X-Ray structural analysis of human ACT containing the P3-P3′ fragment of AT RCL demonstrates helical conformation of the loop, which is not typical for natural ACT and AT [23].

Since the RCL of serpins has no rigid conformation, which is typical for canonical inhibitors interacting with the protease active center via a "lock and key" mechanism [19], serpins can be regarded as substrates, and really are those until the moment when the protease, after the cleavage of the corresponding peptide bond in the loop, becomes covalently attached to a serpin.

The spectrum of ACT mutant forms containing either point P1 mutations or whole insertions up to ten residues into the site of the P1–P1′ bond of AT RCL, including initial variants of these serpins, represented a peculiar set of substrates that in all cases possessed the sequence of RCL residues acceptable for recognition and interaction with duodenase and distinctly different in spatial organization of this loop. In fact, both AT and ACT effectively inhibit duodenase; hence, RCL of both serpins is recognized and cleaved by duodenase, although they contain in their loops at P1 position Leu (ACT), the less preferential in the series of primary specificity of duodenase, and Met (AT), which is virtually not found as the P1 residue in the studied substrates of duodenase. Probably relatively more stabilized spatial structure of the reacting loop of AT compared to ACT better corresponds to the conformational demands of the substrate binding site of duodenase, which can explain the 20-fold higher k_a of duodenase reaction with AT than with ACT. Hence, not only primary structure but also spatial organization of RCL is important for duodenase reaction with serpins. Earlier we have pointed to the domination of tertiary (conformational) component in the substrate specificity of duodenase [4]. The study of duodenase interaction with P1 mutant ACT forms and comparison of the results with similar data for other serine proteases has revealed the interrelation between the role of P1 residue and peculiarity of spatial organization of RCL in efficacy of hydrolytic effect of protease on serpin. Cathepsin G, a structurally related enzyme with similar dual specificity, as well as trypsin and chymotrypsin, were chosen for comparison with duodenase (Table 4). Using kinetic data obtained for interaction of proteases with mutant serpin forms, we compared catalytic efficacy of the proteases in the aspect of primary and tertiary (conformational) specificity. Two varying parameters—P1 residue and RCL conformation—were taken in account in the serpin variants used as substrates. The ratios of association rate constants (k_a) obtained for pairs of inhibitors having either identical RCL type but different residues (Met or Arg) at P1 position or identical P1 residues represented in different types of RCL (AT or ACT) are given in the Table 4.

The indices of primary and conformational specificity were introduced to characterize quantitatively a correlation between the primary and conformational specificity of proteases presented in Table 4.

The indices of primary specificity (Ips and Ips*) characterizing the preferability of one of two P1 residues for given protease (in our case, Met and Arg), represent k_a/k_a ratio for the reaction of the protease and a pair of inhibitors having identical RCL type but different P1 residues. When the conformational RCL type in the pair of inhibitors is less preferable for the protease, Ips is used, whereas Ips* is assigned to more preferential RCL type. So, P1 Arg in ACT is more than 4200 times more effective than P1 Met in the same ACT for trypsin (Ips > 4200). However, this ratio is only 3.2 for AT (Ips* = 3.2).

The index of conformational specificity (Ics) characterizes the preferability of distinct conformational RCL type for protease (AT or ACT in our case) when P1 position in both RCL types contains the same residue. Ics is used for less effective P1 residue, whereas Ics* is used for the more effective one. The conformational type of AT is more preferable than that of ACT (Ics > 1200) for trypsin in the case of P1 Met. When P1 position in RCL is occu-

Table 4. Interrelation of primary and conformational specificity of duodenase, cathepsin G, chymotrypsin, and trypsin in the reaction with AT and mutant forms of AT and ACT containing substitutions at P1 position of the reaction loop. For each pair of the compared inhibitors (P1, residues at P1 position; RCL, serpin loop containing P1 residue), the ratios of the association rate constants (k_a) are given (data of Table 1 are used). Determination of primary and conformational specificity indices (Ips, Ics and Ips*, Ics*, respectively) is described in the text

Specificity indexes	Duodenase			Cathepsin G			Chymotrypsin			Trypsin		
	P1/P1	RCL/RCL	k_a/k_a	P1/P1	RCL/RCL	k_a/k_a	P1/P1	RCL/RCL	k_a/k_a	P1/P1	RCL/RCL	k_a/k_a
Ips	R/M	ACT/ACT	2.5	M/R	AT/AT	5.5	M/R	ACT/ACT	10	R/M	ACT/ACT	>4200
Ips*		no data		M/R	ACT/ACT	20	M/R	AT/AT	10	R/M	AT/AT	3.2
Ics	M/M	AT/ACT	27.9	R/R	ACT/AT	17.2	R/R	AT/ACT	7.6	M/M	AT/ACT	>1200
Ics*		no data		M/M	ACT/AT	62.5	M/M	AT/ACT	7.8	R/R	AT/ACT	1.0
Ics/Ips = = Ics*/Ips*	M/R	AT/ACT	11.2	R/M	ACT/AT	3.1	R/M	AT/ACT	0.77	M/R	AT/ACT	0.3

pied by Arg, both types of RCL are equally effective for trypsin (Ics* = 1).

The Ics/Ips ratio is equal to Ics*/Ips* and characterizes the correlation of conformational and primary specificity of a protease. The Ics value is higher than Ips for duodenase and cathepsin G, thus indicating a dominating role of the conformational component in the substrate specificity of these enzymes. Duodenase has higher Ics/Ips value (11.7) compared with cathepsin G (3.1); hence, conformational requirements of duodenase to substrate are higher than those of cathepsin G, which probably suggests higher selectivity of duodenase for high molecular weight substrates.

The contribution to the specificity of an inhibitor determined by topochemical congruence of the inhibitor structure to the conformational specificity of duodenase is one order of magnitude higher than the contribution of "better" P1 residue with respect to duodenase primary specificity. In connection with this, the cause of apparent inversion of primary specificity of duodenase seems understandable, when the enzyme much more effectively hydrolyzes the P1 Met containing bond in AT than the bond in ACT carrying Arg as the P1 residue (Table 1). As demonstrated, Met does not correspond to primary specificity of duodenase and is virtually never found as P1 residue in substrates hydrolyzed by the enzyme, whereas P1 Arg is one of the most preferable for duodenase [1].

The study on interaction between duodenase and ACT P1 mutants has obviously demonstrated the secondary role of the P1 residue for the inhibition efficacy, when the structure of the inhibitor active site does not correspond to the secondary and conformational specificity of duodenase.

Trypsin and chymotrypsin demonstrate domination of primary over conformational specificity. Higher Ips over Ics value is typical for these proteases. So, Ips/Ics (Ips*/Ics*) = 3.2 for trypsin, and 1.3 for CT (inverse values are given in Table 4).

Thus, the predominance of conformational specificity over primary specificity (Ics > Ips) has been convincingly demonstrated for duodenase and cathepsin G belonging to the special group of granases [4], and, contrastingly, the predominance of the primary specificity (Ips > Ics) for trypsin and CT has been shown.

Low efficiency of interaction between duodenase and chimeric rACT variants containing in their P1–P1′ region of the active loop an insertion of five to ten residues from the corresponding AT reactive loop confirms a supposition on dominating role of conformational specificity of duodenase in its interaction with protein substrates. As we mentioned earlier, chimeric rACT structures have global deformations of RCL due to disturbance of stabilizing bonds between the loop and core of the serpin molecule. The RCL of chimeric serpins represents a "hybrid" of amino acid sequences of AT and ACT RCL, effectively inhibiting duodenase. Theoretically, the primary structure of such loop completely corresponds to primary and secondary specificity of duodenase, which has been demonstrated by calculations [24]. The rACT variant containing the longest insertion P6-P4′, which completely covers the AT loop site in the duodenase active center when enzyme–substrate complex is formed [4] has virtually no interaction with duodenase. Hence, low rate of interaction between such chimeric variants and duodenase can be explained only from the position of mismatch between the conformational state of RCL of these inhibitors and duodenase tertiary structure. In the case of rACT P2-P3′ and rACT P4-P3′, rather effective binding of duodenase and serpin occurs, judging by K_i value, but subsequent proteolysis is hindered, probably

due to the improper positioning of the cleaved bond in relation to the catalytic triad. The substitution of P2-P3′, P3-P4′, and P4-P3′ fragments by the corresponding AT fragments does not change the mobility of distinct residues in ACT active loop, whereas the substitution of P6-P4′ fragment leads to substantial change along the whole loop, as demonstrated by molecular dynamics [21].

Unlike duodenase, chymotrypsin effectively interacts with all serpins studied in the present work, independently of the length of changed RCL fragment. Kinetic parameters of association for CT and a number of chimeric rACT variants with insertions of various lengths (from six to twenty residues) in the reacting loop are given in [25]. Values of kinetic constants suggest virtually equal efficacy of the enzyme interaction with all mutants, independently of the length of changed sequence of reacting loop, thus evidencing a minor role of the conformational component in the substrate specificity of CT compared with duodenase.

The present work has demonstrated that conformational specificity due to the topochemical fitting of the substrate structure to that of substrate-binding site of the enzyme prevails in duodenase and cathepsin G as representatives of the granase group, when recognition and interaction with protein substrate (inhibitor) occurs. The substrate preference of classical proteases of the trypsin group is determined first of all by the P1–S1 substrate–enzyme interactions, that is, by primary specificity of the enzyme.

Thus, a seeming anomaly of enzymatic properties of duodenase in relation to polypeptide substrates (inhibitors) results from the hierarchy of specificity – tertiary (conformational), secondary, primary – with predominance of the conformational component. Conformational selectivity of granases is apparently important in functioning of these proteases *in vivo* as regulatory proteases because it enables fine regulation of the enzyme selectivity to physiological substrates.

We are grateful to Dr. Michael Plotnick for providing the ACT mutant preparations and Dr. Harvey Rubin for collaboration.

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