ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 6, pp. 658-666. © Pleiades Publishing, Inc., 2006.
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Interaction between Human α_2-Macroglobulin and Duodenase, a Serine Proteinase with Dual Specificity

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Received October 25, 2005
Revision received December 15, 2005

Abstract—Interaction between a serine proteinase from bovine duodenum and human serum α_2-macroglobulin (α_2-MG) was studied. α_2-MG is established to be one of the most effective duodenase inhibitors. The enzyme is completely inhibited in less than 30 sec at equimolar ratio of the inhibitor and enzyme (concentration $2 \cdot 10^{-8}$ M). Under identical conditions, the rate of duodenase association with α_2-MG is at least 2.5-fold higher than the rate of chymotrypsin association with this inhibitor. The interaction with duodenase results in proteolysis of the inhibitor subunit in the "bait region". Similarly to other proteases, duodenase in the complex with α_2-MG retains the intact catalytic apparatus and ability to hydrolyze some small substrates. But the duodenase–inhibitor complex is fully inactive to proteins (bovine serum albumin). The stoichiometry of the enzyme interaction with the inhibitor is 2 : 1 (mol/mol). Based on the association rate constant and the termination time of the duodenase and α_2-MG *in vivo* association, α_2-MG is suggested to be a physiological regulator of the enzyme.

DOI: 10.1134/S0006297906060101

Key words: duodenase, α_2-macroglobulin, inhibition, stoichiometry, kinetics

Duodenase, the earlier unknown serine protease of the bovine duodenum, was first isolated and characterized in the Institute of Bioorganic Chemistry, Russian Academy of Sciences [1, 2]. The enzyme was immunocytochemically located in the epithelial cells and secretory ducts of the duodenal (Brunner's) glands and mast cells of the small intestine mucosa [3, 4]. Duodenase is suggested to be a primary activator of the enteropeptidase zymogen, which is a key enzyme in the activation cascade of digestive proteases [5]. Duodenase has been also shown to realize a signaling function and induce synthesis of DNA in pulmonary artery fibroblasts via proteolytic activation of a G-protein-coupled receptor [4].

Duodenase has dual trypsin- and chymotrypsin-like activity [3]. Data of crystallography and computer-aided modeling have shown that the S1-subsite of duodenase (according to the nomenclature presented in [6]) possesses structural features providing for an efficient distribution of P1-residues typical for substrates of both trypsin (Arg/Lys) and chymotrypsin (Tyr/Phe) [7, 8].

Extracellularly active proteases, including duodenase as a secretory protein, are usually physiologically regulated by specific inhibitors. We have been studying for several years the physiological regulation pattern of duodenase activity. Despite the enlarged primary specificity of duodenase, it has a noticeable selectivity to substrates and inhibitors, and this property is specific for regulatory proteases. This feature of the enzyme is caused by the much more pronounced secondary and conformational specificities than those of trypsin or chymotrypsin [8]. Therefore, duodenase is inactive to some substrates and inhibitors formally fitting the requirements of the primary specificity of the enzyme. Thus, the Kunitz- and Kasal-type pancreatic inhibitors (BPTI and PSTI, respectively) functioning in the duodenum and having in the P1-posi-

Abbreviations: α1-AT) α1-inhibitor of proteinases (α1-antitrypsin); α1-ACh) α1-antichymotrypsin; α_2-MG) α_2-macroglobulin; BAEE) N-benzoyl-L-arginine ethyl ester; BBI) soybean Baumann–Birk family proteinase inhibitor; DMSO) dimethylsulfoxide; DTT) dithiothreitol; PEG) poly(ethylene glycol); pNA) *p*-nitroanilide; STI) soybean Kunitz-type trypsin inhibitor; Suc) N-succinyl; Tos) tosyl.

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tion of the inhibitory loop the lysine residue preferential for duodenase are weak inhibitors of duodenase and cannot be its physiological regulators [9]. Serum inhibitors (serpins) α1-AT ($k_{ass} = 2.2 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$) [10] and α1-antichymotrypsin (α1-ACh) ($k_{ass} = 1.1 \cdot 10^4$ $M^{-1} \cdot sec^{-1}$) are more effective toward duodenase (our unpublished data). The purpose of the present work was to study the interaction between duodenase and the serum inhibitor α_2-MG.

Blood plasma contains a large amount of α_2-MG, which is a glycoprotein consisting of four identical subunits with molecular weight of about 185 kD connected in pairs by disulfide bonds [11]. This unique inhibitor is virtually universal and can interact with various endopeptidases, including serine, aspartate, and cysteine proteases, and metalloproteases, independently of their substrate specificity. To ensure productive interaction of α_2-MG and protease, the enzyme has to be catalytically active and hydrolyze one of the inhibitor subunits in the specific bait region located approximately in the middle of the subunit polypeptide chain and containing sites available for hydrolysis by different proteases. Such a limited proteolysis results in significant conformational changes in the inhibitor molecule, activation of thioether groups, and generation of one or two sites for binding proteases [11]. The enzyme falls into a molecular trap, and after the thioether bond, at least one protease molecule covalently binds with the inhibitor by an available lysine residue. In the complex with α_2-MG, proteinases retain their activity to compounds capable of penetrating into the "trap" [11]. The trap closing depends on the size of the seized proteinase and denudes the sites responsible for binding to various cellular receptors. Many functions of the body associated with the blood and lymph circulation systems are regulated by α_2-MG. The liver is the main source of α_2-MG and also the place of its elimination [12]. However, α_2-MG has been also found in the gastrointestinal tract mucosa [13]. The data of the present work on the interaction of α_2-MG and duodenase support the idea that the *in vivo* activity of duodenase can be controlled by this inhibitor.

MATERIALS AND METHODS

The reagents used were as follows: α1-inhibitor of proteinases (α1-AT) from Miles (Canada); soybean Kunitz-type trypsin inhibitor (STI), N-benzoyl-L-arginine ethyl ester (BAEE), bovine serum albumin (BSA), SucAlaAlaProPhe-pNA, and TosGlyProLys-pNA from Sigma (USA); SucThrProLys-pNA synthesized by V. F. Pozdneev (Institute of Biomedical Chemistry, Moscow); chicken ovomucoid (Serva, Germany); trypsin (Merck, Germany); chymotrypsin and Coomassie Brilliant Blue G-250 from Fluka (Switzerland); Sephadex G-200 and Sepharose-6B (LKB Pharmacia, Sweden); poly(ethylene glycol) 300 (PEG) from LOBA Feinchemie (Austria); Tris(hydroxymethyl)aminomethane (Tris), dimethylsulfoxide (DMSO), acrylamide, bis-acrylamide, and dithiothreitol (DTT) from ICN (USA); N,N,N′,N′-tetramethylethylenediamine (TEMED) from Merck (Germany); ammonium persulfate from Reanal (Hungary).

Purification of duodenase. Duodenase was prepared by the modified method described in [1]. The original extract (1.5 liters) of the duodenum mucosa (0.01 M Tris-HCl (pH 8.0), 2 mM EDTA, 1 μM chicken ovomucoid) was successively precipitated in the presence of ammonium sulfate (30% saturation, pH 8.0, and the supernatant was used), in acidic medium (pH 4.0, and the supernatant was used), and with ammonium sulfate (70% saturation, pH 8.0). The resulting precipitate was dissolved in 60 ml of a working buffer (0.05 M Tris-HCl (pH 8.2), 1 M NaCl) and incubated in the presence of an affinity sorbent (STI-Sepharose-6B, 65 ml). The sorbent was placed into a column, washed with the working buffer (6 ml/h) to the corresponding decrease in the optical density ($A_{280} < 0.05$). The active enzyme was desorbed under conditions of a pH gradient (4.0-3.0; 100 ml-100 ml). Fractions containing the active enzyme (substrate TosGlyProLys-pNA) were combined, dialyzed against purified water, and concentrated. To 10 ml of the resulting solution of the protein ($A_{280} = 0.28$) 100 μl of 0.1 M phosphate-citrate buffer (pH 6.0) was added, and the preparation was immediately placed onto a column with a Mono S sorbent (LKB Pharmacia) at the flow rate of 1 ml/min. Then the column was washed for 30 min with 0.01 M phosphate-citrate buffer (pH 6.0). The elution was performed with a NaCl gradient (0-0.3 M) in the same buffer at the rate of 1 ml/min. Fractions 300 μl in volume were collected into tubes containing 30 μl of 0.1 M acetate buffer (pH 3.8). Thus, the pH of the collected fraction was 4.5. The fractions containing the active enzyme were combined and stored at –20°C. The quantity of active sites in the duodenase preparation was determined by titration of the enzyme with a classical soybean Bowman–Birk family inhibitor (BBI) [9]. Here and further activities of proteases were determined with an UV-265 FW spectrophotometer (Shimadzu, Japan) at 25°C.

Isolation of α_2-MG from human blood plasma. Isolation of the inhibitor was performed at 4°C as described in [14] and included three steps: a) successive fractionation of whole plasma (50 ml) with PEG 300 and dissolving the precipitate in 17 ml of saline; b) double precipitation of the proteins with 0.5% rivanol and dissolving the precipitate in 8.5 ml of 3% NaCl; c) gel filtration using Sephadex G-200 (column volume of 370 ml) equilibrated with an elution buffer (0.05 M Tris (pH 7.4), 0.9% NaCl, 0.02% NaN_3). α_2-MG was eluted for approximately 4 h at the flow rate of 21 ml/h. Fractions with maximal contents of α_2-MG (determined on consideration of the trypsin-binding activity of the preparation (see below) and data of electrophoresis under reducing conditions) were frozen and stored at –20°C.

Determination of the active α_2-MG concentration. The active α_2-MG concentration was determined using trypsin and α-chymotrypsin with known active site concentrations [15, 16]. Use of trypsin was based on the α_2-MG quantitative formation with trypsin of a complex capable of cleaving low-molecular-weight substrates but resistant to soybean inhibitor (STI) [17]. For the determination, α_2-MG was supplemented with excess trypsin, and the activity of unbound trypsin was suppressed with STI.

The α_2-MG solution in 0.05 M Tris-HCl buffer with 0.2 M $CaCl_2$ (pH 8.0) was supplemented with 0.1 ml of trypsin solution ($6\cdot10^{-7}$ M) in 1 mM HCl. The mixture was incubated for 10 min at 25°C, supplemented with 0.05 ml of STI (10^{-5} M), incubated for 10 min at the same temperature, and 0.4 ml of the substrate solution (BAEE, $1.5\cdot10^{-3}$ M) in the same buffer was added. The trypsin activity in the complex with α_2-MG was recorded by time-dependent changes in the optical density at 253 nm, with consideration for the spontaneous hydrolysis of the substrate. The active inhibitor concentration was determined by the known trypsin concentration ($6\cdot10^{-8}$ M) and stoichiometry of the interaction with α_2-MG (1 mol enzyme per mol inhibitor [18]).

In the case of α-chymotrypsin, the standardized solution of the enzyme with a constant concentration of $1.57\cdot10^{-8}$ M in 1 mM HCl was supplemented with a varied volume of the inhibitor tested (1.2 mg protein per ml, determined by the Lowry method [19]) and incubated for 10 min at 25°C in 0.68 ml of the incubation medium. Then 0.02 ml of the substrate SucAlaAlaProPhe-pNA (10 mM) solution in DMSO was added. The residual enzymatic activity was determined by the increment of the optical density at 410 nm, taking into account the spontaneous hydrolysis of the substrate. The dependencies of the residual enzymatic activity and also of the free enzyme relative activity (A_{free}/A_0) calculated by the formula (1) [20] on the inhibitor concentration were plotted:

$$A_{free}/A_0 = (a_t - a_{min})/(1 - a_{min}), \quad (1)$$

where A_{free} is activity of the enzyme not bound with α_2-MG, A_0 is the enzyme activity in the absence of the inhibitor, a_t is the total relative activity of the enzyme (bound and not bound with α_2-MG) at the given concentration of the inhibitor, and a_{min} is the relative activity of the enzyme in the complex with α_2-MG. By extrapolation of the linear region, the equivalence point was found (Fig. 1) and based on it the quantity of the active inhibitor was calculated from the stoichiometry of chymotrypsin interaction with α_2-MG at the enzyme concentration of 1.5 mol per mol inhibitor [20]. The active α_2-MG concentration in the preparation was $1.52\cdot10^{-6}$ M.

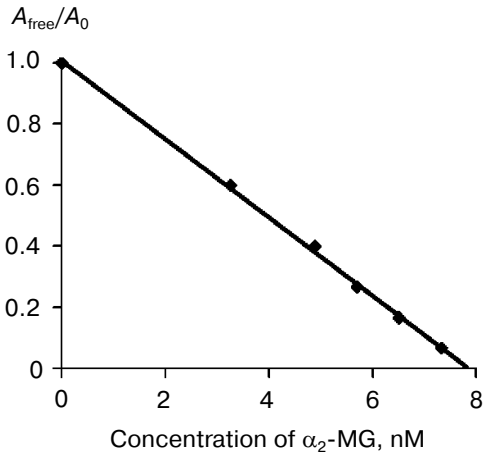

Fig. 1. Determination of the active inhibitor concentration in the resulting preparation of α_2-MG.

The rate constant of chymotrypsin association with α_2-MG was determined under conditions of the second-order reaction. To determine the initial amidase activity of chymotrypsin (A_0), to 0.97 ml of 0.05 M Tris-HCl buffer (pH 8.0) 9 μl of $2.2\cdot10^{-6}$ M chymotrypsin solution in water and 0.02 ml of the substrate SucAlaAlaProPhe-pNA (10 mM) solution in DMSO were added, and changes in the optical density were recorded as described above. To determine the residual activity of the enzyme, into the cuvette to 0.95 ml of the same buffer 9 μl of chymotrypsin solution ($2.2\cdot10^{-6}$ M) and 0.02 ml of α_2-MG solution ($1.52\cdot10^{-6}$ M) in the working buffer were added. The mixture was incubated for 30-300 sec, and then 0.02 ml of the 10 mM substrate SucAlaAlaProPhe-pNA was added. The relative activity of the unbound enzyme, A_{free}/A_0, was calculated by formula (1) [20]. The dependence of the parameter inverse to the relative activity of the unbound enzyme was plotted against the time of incubation with the inhibitor. The rate constant of the chymotrypsin and α_2-MG association was calculated from the equation for the rate of the second-order reaction by formula (2) [21]:

$$A_0/A_{free} = 1 + [E]_0 k_{ass} \cdot t. \quad (2)$$

Determination of the catalytic activity of duodenase in the complex with α_2-MG. The catalytic activity of duodenase and its complex with α_2-MG was determined by the rate of hydrolysis of four substrates: SucAlaAlaProPhe-pNA (substrate 1), SucThrProLys-pNA (substrate 2), TosGlyProArg-pNA (substrate 3), and TosGlyProLys-pNA (substrate 4). Changes with time in the optical density at 410 nm were recorded as described above. To determine the catalytic activity of duodenase in the complex with α_2-MG, equimolar amounts of duodenase and inhibitor (from $1.9\cdot10^{-8}$ M for TosGlyProLys-pNA to $1.9\cdot10^{-7}$ M for the substrate 1) were inhibited for 10 min at 25°C. Then, to inhibit a possible activity of the free enzyme, an excess of α1-AT was added and the incubation

was continued under the same conditions for 15 min; then the corresponding substrate solution was added, and the reaction rate was recorded. The residual enzymatic activity was calculated as the percent of the initial activity.

Kinetic studies on the interaction of duodenase with α_2-MG. To determine the residual activity of duodenase during the incubation with α_2-MG, substrates 1 and 2 were used. Kinetic measurements were performed in 0.05 M Tris-HCl buffer (pH 8.0) at 25°C. The incubation mixture for substrate 1 contained as follows: 0.685 ml of the buffer, 0.01 ml of duodenase solution ($7.2 \cdot 10^{-6}$ M), and 0.075 ml of the inhibitor solution ($1.52 \cdot 10^{-6}$ M). For substrate 2 the incubation medium was as follows: 0.755 ml of the same buffer, 0.01 ml of duodenase solution ($1.7 \cdot 10^{-6}$ M), and 0.015 ml of the inhibitor solution ($1.52 \cdot 10^{-6}$ M). The mixture was incubated for 30-600 sec, and then 0.03 ml of substrate 1 (10 mM) or 0.02 ml of substrate 2 (10 mM) solution in DMSO was added. The zero point corresponded to the initial activity of the enzyme without the inhibitor. Changes with time in the optical density at 410 nm were recorded. The residual activity of duodenase was plotted against the incubation time with the inhibitor.

When substrate 2 was used, the rate constant of duodenase association with α_2-MG was assessed by formula (2) [21].

Determination of stoichiometry of the duodenase interaction with α_2-MG. Stoichiometry of the interaction of duodenase with α_2-MG was determined by suppression of the amidase activity of the protease by the inhibitor after preliminary incubation of the enzyme with the inhibitor in 0.05 M Tris-HCl buffer (pH 8.0) at 25°C for 10 min at strictly defined concentrations of the reagents. The amidase activity of duodenase was determined under standard conditions using substrate 1. The residual enzymatic activity, as well as the relative activity of the free enzyme, was plotted against the inhibitor concentration. The relative activity of the free enzyme was calculated by formula (1). By extrapolation of the linear region, the equivalence point was determined, and based on it the stoichiometry of the process was calculated.

Electrophoretic investigation of the interaction of duodenase with α_2-MG (A) and proteolytic activity of the duodenase complex with α_2-MG (B). The samples were prepared as follows. A) To 20 μl of the α_2-MG preparation ($1.5 \cdot 10^{-6}$ M) in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl, 0.02% NaN_3, and 16 μg of the active inhibitor, varied volumes of duodenase solution were added, which contained 0.2-1.2 μg enzyme and 0.05 M Tris-HCl buffer (pH 8.0), to obtain incubation mixture volume of 23 μl. The mixture was incubated for 10 min at room temperature and then supplemented with 2 μl of DTT solution in water (1.25 M) and 6 μl of the sample buffer. Samples of the inhibitor (16 μg) and duodenase (1.2 μg) reduced in the presence of 1.25 M DTT were used as the control. B) The enzyme (2 μg) solution (5 μl) was mixed with 2 μl of BSA (100 μg) solution in water. To test the proteolytic activity of the duodenase complex with α_2-MG to BSA, 5 μl of duodenase (2 μg) solution was incubated for 10 min at room temperature with 15 μl of α_2-MG (110 μg) solution in buffer (0.05 M Tris-HCl (pH 7.4), 0.9% NaCl, 0.02% NaN_3), and then 2 μl of BSA (100 μg) solution in water was added. As the control, 2 μl of BSA (100 μg) solution, 5 μl of duodenase (2 μg) solution, and a mixture of the same quantities of BSA and duodenase were used. All samples were supplemented with the due amount of the working buffer (0.05 M Tris-HCl (pH 8.0)) to the volume of 22 μl. All samples were incubated for 41 h at 37°C. Before application onto the plate, all samples were supplemented with 5 μl of 1.25 M DTT solution and 6 μl of the fivefold buffer for application, and then the samples were kept for 20 min at 80°C and centrifuged.

SDS-electrophoresis by the Laemmli method [22] was performed using a Mini-Protean II apparatus (Bio-Rad, USA) in 12 and 15% gels at 20°C for 40 min at 200 mV. The gels were stained for 15 min in a solution containing 0.15% Coomassie Brilliant Blue R-250, 30% methanol, and 10% acetic acid, and washed for 24 h in a solution containing 10% methanol and 10% acetic acid.

Densitometric scanning. The gel was scanned with a Mustek 12000 SP scanner, the data were processed with a PC using the One-Dimensional Gel Analysis program, One-Dscan, version 1.3, the area under the peak corresponding to the 90-kD band was calculated, and the peak area (as percent of the maximum) was plotted against the ratio of enzyme/inhibitor.

RESULTS

Determination of the catalytic activity of the duodenase–α_2-MG complex toward low-molecular-weight and protein substrates. The activities of enzyme complexes with α_2-MG to substrates with different molecular weight and structure can vary, because during the α_2-MG interaction with a proteinase the active site of the latter remains free but sterically blocked by the inhibitor molecule. Proteinases in the complex with α_2-MG retain 100% activity to various low-molecular-weight synthetic substrates [18]. We have studied the rate of hydrolysis of four synthetic substrates 1, 2, 3, and 4 (see "Materials and Methods") and BSA by the duodenase complex with α_2-MG. In the presence of α_2-MG, the rate of the duodenase-catalyzed hydrolysis only of substrates 1 and 2 was decreased (Fig. 2). The residual rate of their hydrolysis was 45 and 20%, respectively, of the hydrolysis in the absence of α_2-MG.

The rate of hydrolysis of two other substrates by duodenase did not change in the presence of α_2-MG. Addition to the α_2-MG complex with duodenase of α1-AT (which rapidly suppressed the free duodenase activity

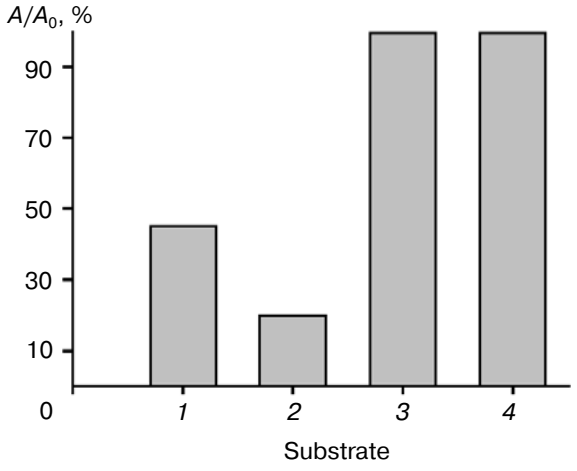

Fig. 2. Relative activity of duodenase (A/A_0) in the complex with α_2-MG with respect to different substrates: *1*) SucAlaAlaProPhe-pNA; *2*) SucThrProLys-pNA; *3*) TosGlyProArg-pNA; *4*) TosGlyProLys-pNA.

[10]) did not decrease the rate of hydrolysis of substrates 1 and 3. These findings indicated that the active site of duodenase completely retained the catalytic activity in the complex with α_2-MG, and the decrease in the rate of hydrolysis of some low-molecular-weight synthetic substrates by duodenase in the complex with α_2-MG was caused by steric factors. With BSA as an example, the complex of duodenase with α_2-MG was shown to be absolutely inactive to high-molecular-weight proteins (Fig. 3, a and b).

Kinetics of the interaction of duodenase with α_2-MG. Figure 4 shows the decrease in the duodenase activity toward hydrophobic and charged substrates in dependence on the time of interaction with α_2-MG in comparison with the kinetic curve of chymotrypsin inhibition. When substrates 1 and 2 were used, duodenase was virtually completely complexed with the inhibitor after 30 sec (the minimum incubation time). The duodenase concentration of 20 nM used for assessment of the association rate constant was the least concentration which allowed the enzyme activity to be detectable under the experimental conditions (with substrate 2). Under similar conditions, chymotrypsin was fully inhibited for about 70-80 sec (Fig. 4). Thus, the rate of duodenase association with α_2-MG was, at least, twofold higher than this value for chymotrypsin. Under our conditions, the k_{ass} of chymotrypsin with α_2-MG was $6.5 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$, which was approximately twofold lower than the value known from the literature ($\geq 1.2 \cdot 10^7$ $M^{-1} \cdot sec^{-1}$ [23]). The lower limit of the k_{ass} value for duodenase association with α_2-MG determined by formula (2) was $1.6 \cdot 10^7$ $M^{-1} \cdot sec^{-1}$. Table 1 presents rate constants of the association of α_2-MG with some serine proteinases. According to our classification of chymotrypsin family proteases, two groups can be distinguished depending on specific features of the active site: the trypsin group and the granase group [24]. Association rate constants of the 10^7 order are characteristic for proteases of the trypsin group with molecular weight lower than 30 kD (trypsin, chymotrypsin, elastase of neutrophils). Kinetic parameters of interaction with α_2-MG are known (except duodenase) only for two granases: chymase of mast cells and human cathepsin G. Both these enzymes interact with α_2-MG at nearly the same rates and, at least, 3.2- and 4.3-fold, respectively, slower than duodenase.

Stoichiometry of the interaction of duodenase with α_2-MG was determined by titration of the enzyme at its concentration of $1.7 \cdot 10^{-7}$ M by the inhibitor using substrate 1. The dependence of the relative activity of duodenase on the inhibitor/enzyme ratio is presented in Fig. 5a. One can see that at the ratio $[I]_0/[E]_0 = 0.6$ the enzyme

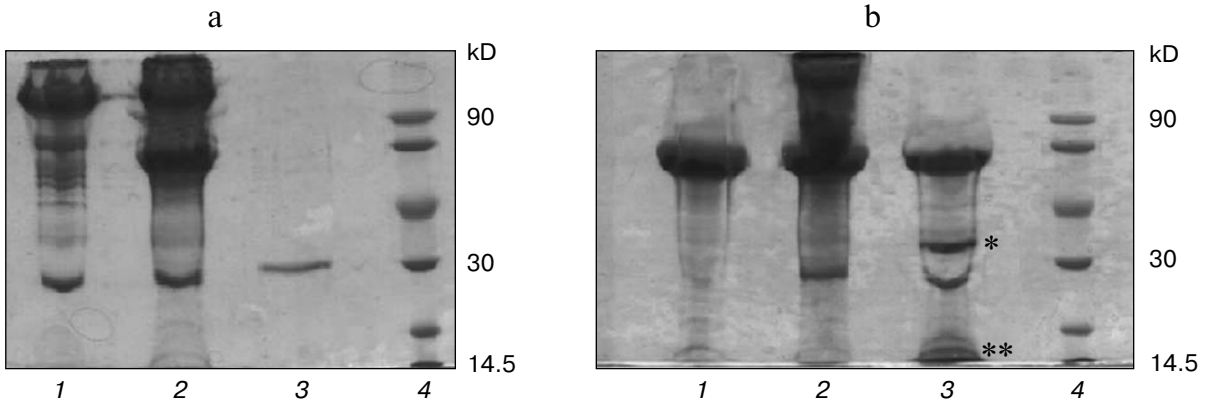

Fig. 3. Interaction of duodenase (free and in the complex with α_2-MG) with BSA (SDS-PAGE in 15% polyacrylamide gel under reducing conditions). All samples were incubated at 37°C for 41 h. a: *1*) original α_2-MG; *2*) BSA + α_2-MG; *3*) duodenase; *4*) markers of molecular weight. b: *1*) BSA; *2*) BSA + duodenase–α_2-MG complex; *3*) BSA + duodenase (the asterisks indicate proteolysis products); *4*) markers of molecular weight.

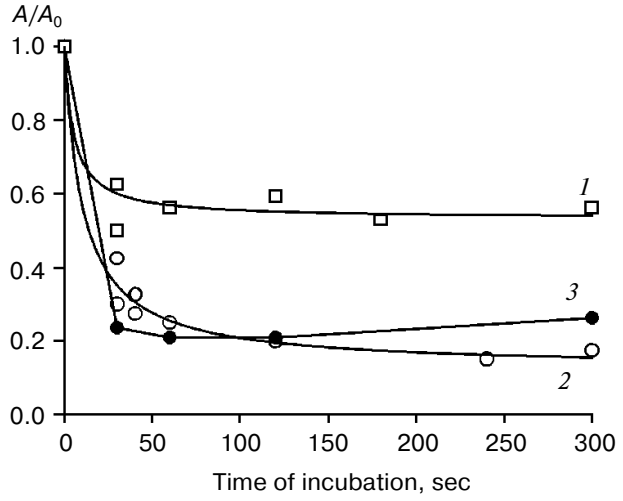

Fig. 4. Kinetic curves of the interaction of α_2-MG with duodenase and chymotrypsin: *1*) duodenase, substrate 1, [E] = [I] = 10^{-7} M; *2*) chymotrypsin, substrate 1, [E] = [I] = $2 \cdot 10^{-8}$ M; *3*) duodenase, substrate 2, [E] = [I] = $2 \cdot 10^{-8}$ M; 25°C, 0.05 M Tris-HCl (pH 8.0).

activity decreases to 45%, which corresponds to the activity of the complex (see above), and it then remains unchanged. The free enzyme activity was calculated by formula (1). The dependence of the relative activity of free duodenase on the inhibitor/enzyme ratio is presented in Fig. 5b. At the ratio $[I]_0/[E]_0 = 0.6$ the free enzyme activity was nil. Thus, at the enzyme concentration of $1.7 \cdot 10^{-7}$ M the stoichiometry of the interaction (SI) was approximately 2 mol duodenase per mol α_2-MG, which indicated the binding of another enzyme molecule to another free active site of the inhibitor. The stoichiometry of the interaction of α_2-MG was studied in the narrow range of concentrations ($(2\text{-}3) \cdot 10^{-7}$ M) with other serine proteases: bovine trypsin (SI 0.6), chymotrypsin (SI 0.8), and human chymase (SI 0.9) [20]. Thus, the stoichiometry of the interaction of duodenase with α_2-MG is the same as that of trypsin determined under similar conditions. It has been shown [27] that the interaction of α_2-MG with proteinases depends on their size and, respectively, molecular weight. For proteinases with molecular weight lower than 30 kD (the molecular weight of duodenase is 26.5 kD) the stoichiometry is, as a rule, 2 mol enzyme per mol inhibitor (at sufficient concentration of the enzyme [27]), and in this case rate constants of association are high (about 10^7-10^6 $M^{-1} \cdot sec^{-1}$) and correspond to the primary hydrolysis in the bait region. The other molecule of protease is bound after significant conformational changes in the inhibitor molecule initiated by the primary cleavage, and this binding is accompanied by hydrolysis of the bait region of the other subunit; this secondary cleavage is characterized by a significantly lower rate and, respectively, one-two orders lower values of k_{ass}. Proteinases with higher molecular weight (thrombin, kallikrein) have low values of k_{ass} (about 10^4 $M^{-1} \cdot sec^{-1}$), and the stoichiometry of the interaction with the inhibitor more often is 1 : 1. This is caused by steric obstacles to the binding of large molecules and a decreased availability of the bait region for such proteinases. The rate of the bait hydrolysis and, respectively, kinetic parameters of the interaction with α_2-MG depend on the protease specificity. The amino acid sequence of this proteolysis-sensitive region of the inhibitor contains hydrolysis sites, which include nearly the whole spectrum of the protease specificities, but regulatory enzymes with pronounced secondary and tertiary specificities hydrolyze the α_2-MG bait region more slowly. This explains the lower values of k_{ass} for the interaction with the inhibitor of such proteases as cathepsin G and mast cell chymase, although the molecular weights of these enzymes are close to that of trypsin. But duodenase is a kind of exception, because, along with its noticeable secondary and tertiary specificities similar to other granases [8], it interacts with α_2-MG with a very high rate. It is suggested that, due to its dual specificity to the P1 residue, duodenase can recognize in the bait region hydrolysis sites specific for both trypsin and chymotrypsin, and this finally increases the rate of hydrolysis by duodenase of the corresponding region of the inhibitor.

Study on the interaction of α_2-MG with duodenase by SDS-PAGE. The interaction of duodenase with α_2-MG was studied by SDS-PAGE under reducing conditions at varied enzyme/inhibitor ratios. The results are shown in Fig. 6a. The α_2-MG concentration was constant, where-

Table 1. Rate constants of association of α_2-MG (binding the first molecule of the enzyme) with some serine proteases

Protease	k_{ass}, $M^{-1} \cdot sec^{-1}$	Reference
Duodenase	$>1.6 \cdot 10^7$	
Cathepsin G	$(2.5\text{-}5.1) \cdot 10^6$	[25]
Elastase (of neutrophils)	$4.1 \cdot 10^7$	[25]
α-Chymotrypsin	$6.5 \cdot 10^6$ $1.2 \cdot 10^7$	[23]
Trypsin	$2 \cdot 10^7$	[26]
Human mast cell chymase	$5 \cdot 10^6$	[20]
Plasmin	$5 \cdot 10^5$	[26]
Human plasma kallikrein	$(1.8\text{-}2.8) \cdot 10^4$ (23°C) $(4.1\text{-}5.3) \cdot 10^4$ (37°C)	[26]
Thrombin	$<1 \cdot 10^4$	[27]

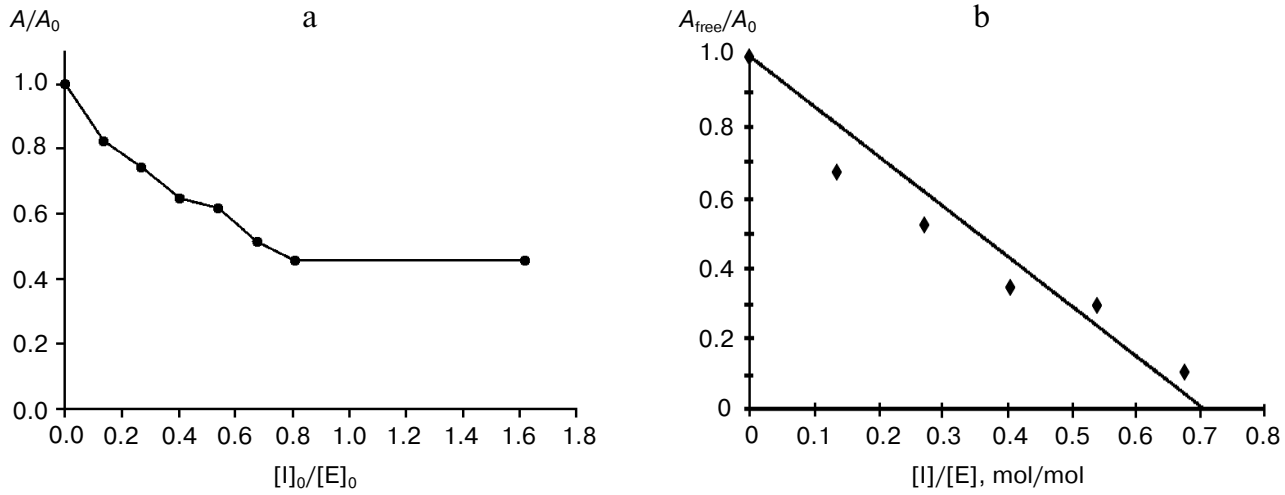

Fig. 5. Determination of stoichiometry of the interaction of duodenase with α_2-MG. Substrate 1, [E] = $1.7 \cdot 10^{-7}$ M. The dependence of the relative total activity of the enzyme (a) and the relative activity of the free enzyme (b) on the $[I]_0/[E]_0$ ratio.

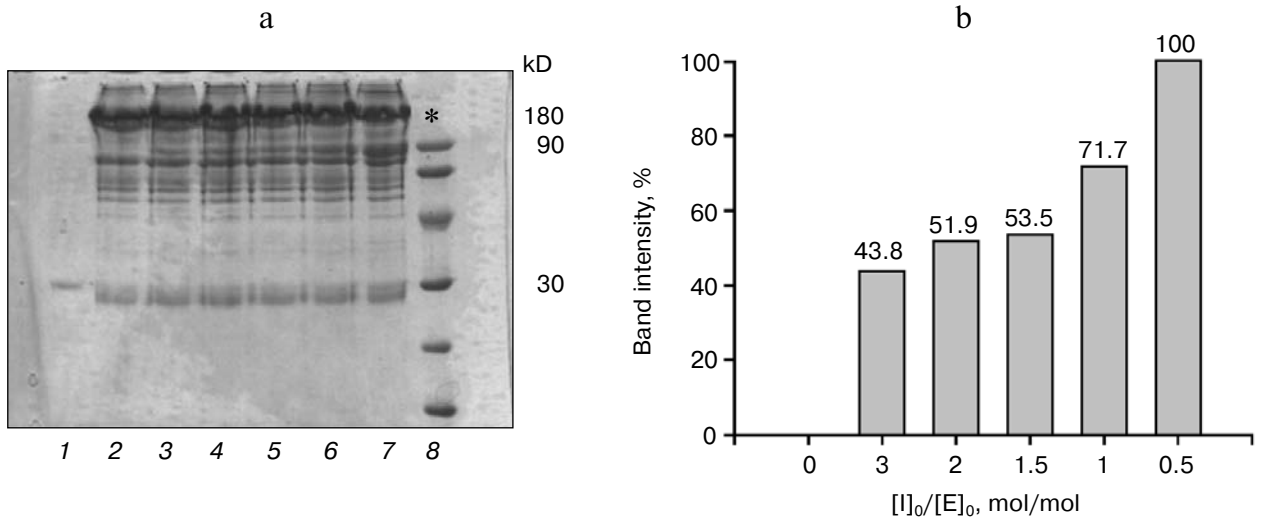

Fig. 6. a) Interaction of duodenase and α_2-MG at varied ratios of the enzyme and inhibitor concentrations (SDS-PAGE in 12% polyacrylamide gel under reducing conditions): *1*) duodenase (1.2 μg); *2*) α_2-MG (16 μg); *3-7*) enzyme and inhibitor at $[I]_0/[E]_0$ ratio of 3, 2, 1.5, and 0.5, respectively; *8*) markers of molecular weight. b) Area of the peak (in percent of maximum) obtained by densitometric scanning of the band with molecular weight of ~90 kD against the enzyme/inhibitor ratio.

as the enzyme concentration was varied. The α_2-MG subunit moved during SDS-PAGE with the rate that corresponded to molecular weight of about 180 kD. With the decrease in the $[I]_0/[E]_0$ ratio, the electrophoregram presented an increase in the intensity of a band in the region of 90 kD. Figure 6b shows results of densitometric scanning of the 90 kD band. The appearance of the band in this region was caused by cleavage by duodenase of specific bonds in the bait region of α_2-MG and the resulting detachment of the N-terminal part of the inhibitor subunit [11].

DISCUSSION

The present work continues our studies on the interaction of duodenase and serum inhibitors of proteases designed to determine the mechanism of natural regulation of duodenase. Duodenase is structurally similar to immunocompetent cell proteinases, such as chymases and granzymes [24], but as a dominant proteinase of the duodenal gland secretion, it occurs in the duodenum lumen where it seems to be directly involved in the regulation of digestion [3]. The location of duodenase in such

Table 2. Interaction of duodenase with human plasma inhibitors

Inhibitor	Physiological concentration, μM	k_{ass}, $M^{-1}\cdot sec^{-1}$	$t_{99\%}$ *in vivo*, sec
α1-ACh	4-8 [21]	$1.1 \cdot 10^4$ (our unpublished data)	57-114
α1-AT	20-70 [21]	$2.2 \cdot 10^5$ [10]	0.3-1.14
α_2-MG	2-6 [11]	$>1.6 \cdot 10^7$	0.05-0.16

different cells as epitheliocytes of Brunner's (duodenal) glands and mast cells suggests the multiplicity of duodenase functions. The physiological role of duodenase is supposed to be related with processing of proenteropeptidase, which is a precursor of the key enzyme of the activation cascade of digestive proteinases [5]. Moreover, duodenase can be involved in tissue remodeling due to its ability to initiate mitosis of fibroblasts with involvement of proteolytic activation of the PAR family receptors [4]. Functional properties of duodenase are in many aspects similar to those of some mast cell proteases, which are sensitive to serum inhibitors (serpins, α_2-MG). The probability of appearance of these inhibitors in the duodenum mucosa is rather high. Apart from conditions of disease (when serum inhibitors can penetrate into the mucosa because of the increased permeability of capillaries), the autonomous presence of some serpins in the secretion of duodenal glands [29] and α_2-MG in the small intestine mucosa [13] have been shown.

We earlier determined kinetic parameters of the interaction of duodenase with two inhibitors from human blood plasma: α1-AT and α1-anti-chymotrypsin (α1-ACh). Kinetic parameters describing the interaction of proteinases with their protein inhibitors determined in the *in vitro* systems can be used for prediction of the *in vivo* efficiency of these inhibitors. There is an approach for *in vivo* assessment of the effect of proteinase inhibitors [30]. This approach is based on the pseudo-first order of the corresponding reactions in the body, because the concentration of proteinases is significantly lower than the concentration of inhibitors. Consequently, the time of termination of the association ($t_{99\%}$) will be $t_{99\%} = 5/(k_{ass}\cdot[I]_0)$, where k_{ass} is the rate constant of the second-order association and $[I]_0$ is the inhibitor concentration *in vivo*.

Table 2 presents the rate constants of duodenase association with the human blood plasma inhibitors studied by us, as well as the calculated time $t_{99\%}$.

Under conditions of disease, in particular, inflammation, when destruction of the duodenum tissues occurs or the permeability of capillaries is increased, duodenase can make direct contact with blood plasma. We can use our kinetic data to assess the efficiency of proteolysis suppression by duodenase in the complex with α_2-MG. The α_2-MG concentration in blood plasma is 2-6 μM [11], and, respectively, the time of completion of the duodenase association with α_2-MG is, at least, six-fold less than the time of duodenase inhibition by α1-AT and 600-fold less than the time of its inhibition by α1-ACh.

Thus, evaluation of the time of the complete association of duodenase with α_2-MG *in vivo* and the rate constants of association suggests that α_2-MG is the best of the duodenase inhibitors studied by us and seems to be a physiological regulator of the enzyme, in addition to α1-AT.

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