MECHANISM OF PROTEINASE COMPLEX FORMATION WITH α_2-MACROGLOBULIN

Three modes of trypsin binding

Lars SOTTRUP-JENSEN, Torben E. PETERSEN and Staffan MAGNUSSON
Department of Molecular Biology, University of Aarhus, 8000 Aarhus C, Denmark

Received 30 March 1981

1. Introduction

The tetrameric plasmaglycoprotein α_2-macroglobulin (α_2M), M_r 725 000, forms complexes with proteinases from all 4 classes (EC 3.4.21–24) [1–3] that retain proteolytic activity towards small substrates [4–6]. Complex formation is initiated by proteolytic cleavage in the 'bait' region near the middle of the 4 identical M_r 180 000 subunits [1–3,7–9] at sites, which reflect the specificity of the particular proteinase [10]. The initial complex is rearranged into the final α_2M–proteinase complex which involves different binding sites on both α_2M and the proteinase [10]. It has been suggested [9,11] that α_2M may bind more trypsin molecules than the 2/mol α_2M that can be protected from inhibition by STI [8,9,12,13].

As shown in [13] α_2M contains a thiol ester in each of its 4 subunits. This thiol ester involves the γ-carboxyl group of a Glx-residue and the sulfhydryl group of a Cys-residue, located as residues ~472 and ~469, respectively, from the C-terminus of α_2M [14]. During complex formation with proteinases the thiol esters are cleaved, resulting in the appearance of free SH-groups [13]. 'Inactivation' of the proteinase-binding capacity of α_2M by CH_3NH_2 also leads to cleavage of the thiol esters [13]. During this process CH_3NH_2 reacts covalently with the Glx-residues to form 4 residues of γ-glutamylmethylamide at complete 'inactivation' [13–15]. Since methylamine and putrescine readily become covalently incorporated in nascent α_2M–trypsin complex [16] it appears that activated α_2M can participate in 'acyl transfer' reactions [13,16], which has also been suggested for the complement components C3 [17–21] and C4 [22]. The complexes that α_2M forms with proteinases may be covalent [10,13,23–25] and other proteins present during activation of α_2M by proteinases may to some extent be simultaneously incorporated [27] as with TLCK–trypsin and insulin in [16].

Here, we describe a model for the interaction of α_2M with trypsin and show that α_2M can bind trypsin in 3 distincts modes. At binding ratios of $\leqslant$2 mol trypsin/mol α_2M all of the trypsin is bound through sites not involving its active site leading to protection against inhibition by STI. Under these conditions 56–67% of the complexed trypsin is bound covalently to α_2M in the sense that it is not dissociated by gel-filtration in 6 M guanidine–HCl after reduction and alkylation while the rest is non-covalently bound. Additional trypsin can be bound (presumably up to 2 mol/mol α_2M) by interaction between the active site of trypsin and the 'bait' region of α_2M [10]. We also show that CH_3NH_2 can compete with trypsin for the covalent binding to α_2M.

2. Materials and methods

Human α_2M was prepared by Zn^{2+}-affinity chromatography as in [13]. Bovine trypsin was purified using a CHOM-column as in [16,27]. The preparation was 88% active and only β-trypsin was seen on SDS–PAGE. 30 mg was iodinated with $Na^{125}I$ (spec. act. 15.5 Ci/mg I) (The Radiochemical Centre, Amersham) using the chloramine-T procedure [28]. Following gel

Abbreviations: α_2M, α_2-macroglobulin; SDS–PAGE, sodium dodecyl sulphate–poly-acrylamide gel electrophoresis; CHOM, chicken ovomucoid; PTI bovine pancreatic trypsin inhibitor (Kunitz); DTT, dithiothreitol; STI, soybean trypsin inhibitor; BAPNA, N^{α}-benzoyl-L-arginine-*p*-nitroanilide

filtration on Sephadex G-25 (Pharmacia) in 50 mM CH_3COONH_4 (pH 4.5) and dialysis against 1 mM HCl the ^{125}I-labelled trypsin (mixture of β- and α-trypsin, 76% active) was freeze-dried. The specific activity was 1.06×10^7 cpm/mg protein as determined in an LKB-Wallach Ultrogamma counter (Bromma). PTI was obtained from Novo (Bagsværd). The concentration of α_2M was determined using $E^{1\%}_{280} = 9.1$ [29] and M_r 725 000 [30]. $CH_3NH_2 \cdot HCl$ was from Sigma (St Louis MO), iodoacetamide, BAPNA and guanidine–HCl from Merck (Darmstadt). DTT was from Fluka (Buchs). Samples of α_2M–trypsin complex that were subjected to gel-chromatography on Sephacryl S-300 had been prepared from stock solutions of α_2M (15.0 μM in 0.05 M Na-phosphate, 0.1 M NaCl (pH 8.0)) and ^{125}I-labelled trypsin (161 μM in 1 mM HCl). The stock solutions of α_2M and trypsin (52% active) used for assay of trypsin protected from inhibition by STI were 13.5 μM and 106 μM, respectively. STI was 227 μM in 0.1 M Na-phosphate (pH 8.0) (buffer A). DTT and iodoacetamide were 0.5 M in water. PTI was 1.54 mM in buffer A. Buffer B consisted of 0.5 M Tris–HCl, 6 M guanidine–HCl (pH 8.0).

3. Experimental and results

The complexes between α_2M and trypsin formed under the conditions given in fig.1,2 were subjected to gel chromatography under non-denaturing (fig.1) and denaturing (fig.2) conditions. The results shown in fig.1,2 represent the '○' and the '●' experimental points, respectively, for [active trypsin]/[α_2] = 2.14 in fig.3. Fig.3 shows the molar ratio of trypsin bound (active plus inactive) to α_2M at varying concentrations of active trypsin added under different incubation and elution conditions. The initial slope of (×) and (○), representing trypsin bound to α_2M in the absence of CH_3NH_2, with or without subsequent addition of PTI, corresponds to 1.07 mol trypsin bound/mol active trypsin. The maximal amount of ^{125}I-labelled trypsin that remained bound after addition of PTI was 2.25 mol/mol α_2M (○).

The curve (×) shows that α_2M can bind additional trypsin in the absence of PTI. At 4.28 mol active trypsin/mol α_2M, 3.54 mol trypsin/mol α_2M was bound under these conditions. At all incubation levels >2 mol trypsin/mol α_2M the ^{125}I-label was found either in the position of α_2M or trypsin (or its autolysis products) (fig.1), indicating a relatively strong interaction between α_2M and trypsin at these sites.

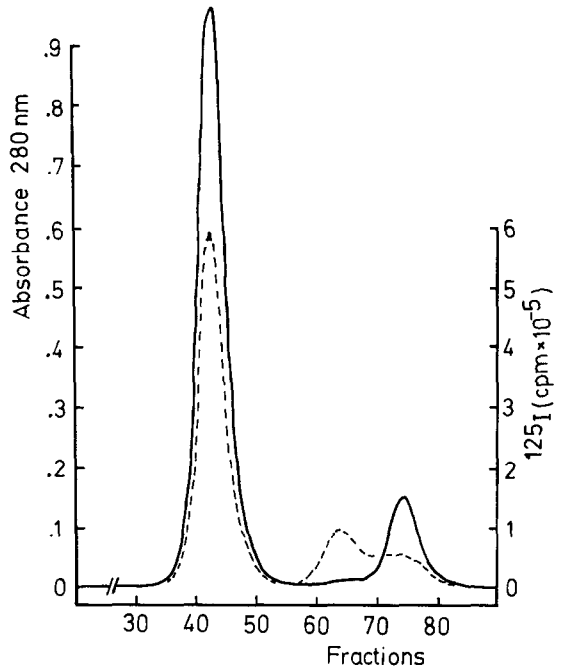

Fig.1. Representative gel chromatography of α_2M treated with ^{125}I-labelled trypsin on Sephacryl S-300 (1 × 94 cm) in buffer A (non-denaturing conditions). Flowrate 4.0 ml/h; fraction size 1.0 ml. A 500 μl sample of α_2M was incubated with 100 μl ^{125}I-labelled trypsin and 300 μl buffer A for 2 min at room temperature. [active trypsin]/[α_2M] = 2.14. Then 100 μl PTI was added and the mixture was loaded on the column. The absorbance at A_{280} nm (——) and the radioactivity (– –) of each fraction was determined. The recovery of both absorbance and radioactivity was 92%. The range of recovery for all experiments (~25) was 88–95%. The amount of trypsin bound to α_2M was determined by multiplying the total amount of trypsin (active plus inactive) with the fraction of radioactivity associated with the α_2M peak. α_2M–trypsin–PTI ternary complex was eluted in tubes 35–52, trypsin–PTI complex in 58–68 and PTI and low molecular mass autolysis products of trypsin in tubes 68–80. A control experiment with trypsin alone showed that ~10% of the ^{125}I-label of trypsin was eluted in the 68–80 position.

The presence of 0.13 M CH_3NH_2 decreases the amount of trypsin bound to α_2M by 2–5% (fig.3 (□)). The results of gel chromatography following reduction and alkylation of α_2M–trypsin complex mixtures show that 56–67% of the trypsin bound firmly under the non-denaturing conditions (fig.1) is still bound to α_2M-fragments even under the denaturing conditions of fig.2 (fig.3 (○)). As evident from fig.3 (■) the amount of trypsin bound to α_2M in the presence of 0.13 M CH_3NH_2 had decreased by ~50% when examined after reduction and alkylation.

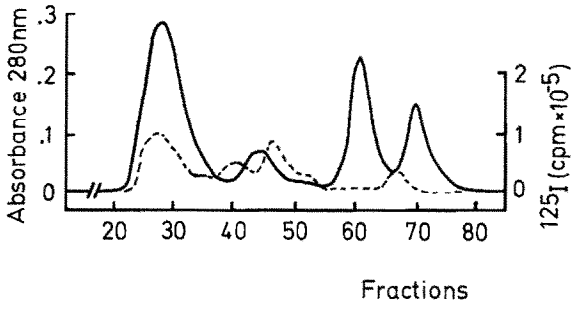

Fig.2. Representative chromatography of α_2M treated with ^{125}I-labelled trypsin on Sephacryl S-300 (1 × 93 cm) in buffer B (denaturing conditions). α_2M (250 µl) was incubated with 50 µl ^{125}I-labelled trypsin and 100 µl buffer A for 2 min. Then 50 µl PTI was added followed by 100 µl 1 M Tris–HCl, (pH 8.5), 500 mg guanidine–HCl and 20 µl DTT solution. After reduction for 1 h at room temperature 50 µl ICH_2CONH_2 was added. Following alkylation for 10 min the sample was loaded on the column (both equilibrated and eluted with buffer B). Flowrate 4.0 ml/h; fraction size 1.3 ml. Absorbance at 280 nm (——); radioactivity (– – –). The recoveries of both protein and radioactivity were ~90%. Tubes 23–33 apparently contained trypsin bound to α_2M-fragments and 34–54 unbound β-trypsin and fragments derived from α-trypsin. Tubes 55–65 contained PTI. Low molecular mass autolysis products from trypsin were eluted in tubes 65–70 and DTT in tubes 66–76. The material in tubes 40–48 probably represents fragments of α_2M derived as a result of slight digestion with trypsin.

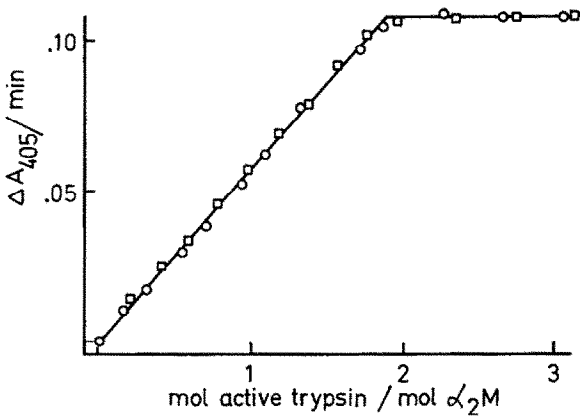

Fig.4. Titration of trypsin bound to α_2M and protected from inhibition by STI in the absence (○) or presence (□) of 0.13 M CH_3NH_2. For experiments performed in the absence of CH_3NH_2 100 µl α_2M (13.5 µM) was mixed with 150–72 µl buffer A. After incubation for 60 s with 0–39 µl trypsin (106 µM), 0–39 µl STI (227 µM in buffer A) was added. After incubating 120 s a 100 µl aliquot was removed and added to a cuvette containing 50 µl BAPNA (20 mM in dimethylsulfoxide) and 1850 µl buffer A. The increase in absorbance at 405 nm was recorded for 120 s. For experiments performed in the presence of CH_3NH_2 80–42.5 µl buffer A was mixed with 27.5 µl CH_3NH_2 (1 M in buffer A) followed by addition of 100 µl α_2M. After incubation for 10 s, 2.5–40 µl trypsin was added. After further incubation for 60 s, 2.5–40 µl STI was added followed by 37.5–0 µl buffer A. Then a 100 µl aliquot was removed after 120 s and assayed as above. All incubations and assays were done at room temperature (22–23°C).

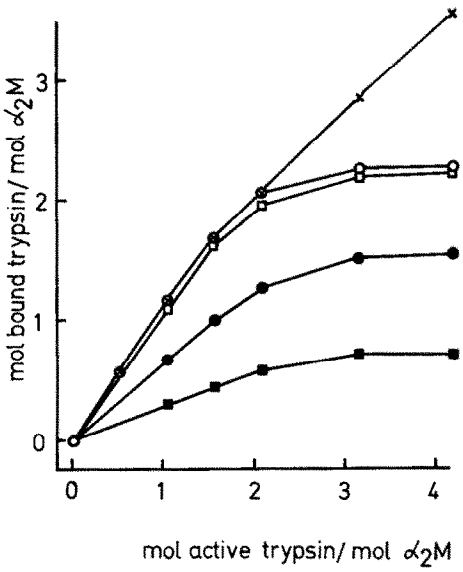

Fig.3. Binding of ^{125}I-labelled trypsin to α_2M at different concentrations of trypsin under non-denaturing and denaturing conditions. Mixtures of α_2M (500 µl or 250 µl) with varying amounts of ^{125}I-labelled trypsin (final vol. 1000 µl or 500 µl, respectively) had been subjected to gel chromatography in buffer A (fig.1). Samples were prepared in the absence (○) or the presence (□) of 0.1 M CH_3NH_2. Parallel samples prepared in the absence (●) or the presence (■) of 0.13 M CH_3NH_2 were reduced, carboxamidomethylated and subjected to gel chromatography in buffer B (fig.2). Samples were also prepared in the absence of CH_3NH_2 and PTI and subjected to gel chromatography in buffer A (×). An additional point (2.97 mol trypsin bound/ mol α_2M for 8.56 mol active trypsin/mol α_2M) is not shown here.

Fig.4 shows that the amount of trypsin protected from inhibition by STI is 1.88 mol/mol α_2M regardless of whether the α_2M–trypsin complexes were formed in the absence or the presence of 0.13 CH_3NH_2.

4. Discussion

As shown in [13] the cleavage of the thiol esters of α_2M shows a 4:1 stoichiometry between appearance of SH-groups and trypsin at <1 mol trypsin/mol α_2M. Under these conditions it appears that cleavage of two 'bait' region sequences is required for binding one trypsin molecule to α_2M in a mode resulting in protection against STI [7–9]. Thus only 1/2 of the thiol ester cleavage is strictly correlated with 'bait' region cleavage [13]. Complete cleavage of all 4 'bait' regions occurs at >2 mol trypsin/mol α_2M resulting in appearance of 4 mol SH/mol α_2M [13]. Furthermore, maximally 2 mol trypsin/mol α_2M can be protected against inhibition by STI [7–9,13]. These results indicate that the cleavage reactions in α_2M leading to formation of protected α_2M–trypsin complexes take place in at least 2 steps, namely reaction (1):

α_2M + trypsin $\rightleftarrows$ α_2M · trypsin

(cleavage of 2 'bait' regions and 4 thiol esters)

and reaction (2):

α_2M · trypsin + trypsin $\rightleftarrows$ α_2M · trypsin$_2$

(cleavage of the remaining 2 'bait' regions).

Fig.5 shows a schematic model of the reaction paths in the formation of α_2M–trypsin complexes, compatible with the available evidence. Prior to cleavage in the 'bait' region trypsin and α_2M form a non-covalent complex (binding mode I) engaging the active site of trypsin and the 'bait' region of one of the subunits of α_2M. Since the tetrameric α_2M is assembled from 2 'half molecules' of M_r 360 000, each consisting of 2 disulfide-bridged M_r 180 000 subunits [30] and since α_2M can bind anhydrotrypsin reversibly (2 mol/mol α_2M) [26] it is likely that the 'half molecules' are the functional units of α_2M.

Following initial non-covalent complex formation one of the possibly two reversibly bound trypsin molecules initiates the activation of α_2M by cleaving the 'bait' region sequence in 2 subunits at a specific Arg–Leu bond [10]. This triggers a conformation change in α_2M [7,9] affecting all 4 subunits and rendering the thiol ester in each subunit of α_2M susceptible to nucleophilic attack [13]. This process generates 'nascent' α_2M–trypsin complex which exists only for a short time [16]. Accompanying the conformational change the first trypsin molecule, already positioned due to the interaction between its active site and the 'bait' region of α_2M, is entrapped within the 'half molecule'. The trypsin thus entrapped is now bound in its final binding site, involving sites on α_2M different from the 'bait' region and sites on trypsin different from the active site [10].

Since the conformational change in α_2M is effected by the first trypsin to cleave α_2M it follows that the second trypsin, presumably also positioned at the 'bait' region of one of the remaining 2 subunits is like-

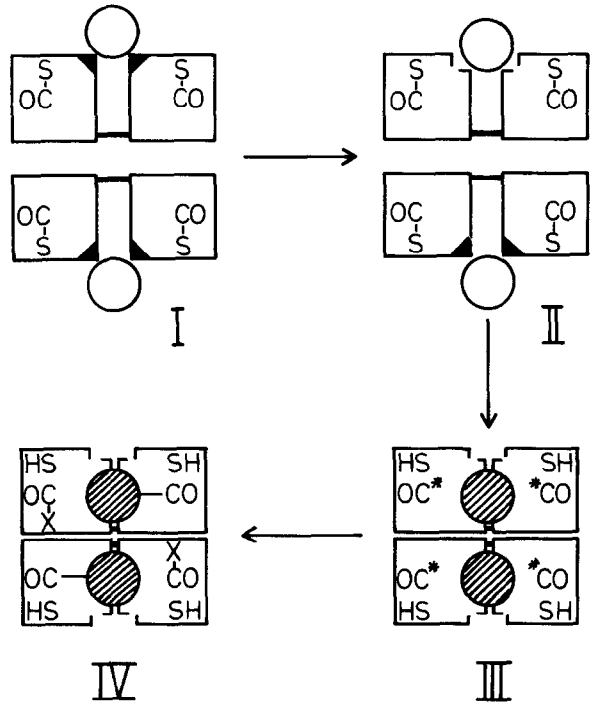

Fig.5. Schematic model of the reaction paths in the formation of α_2M–trypsin complexes. The 'half molecules' of α_2M consisting of two disulfide-bridged subunits are represented by pairs of squares, connected by a thick solid line. The 4 square assembly can exist in 2 states: an open form (native α_2M) and a compact form (α_2M–trypsin complex). The 'bait' region is either represented by a filled triangle at one corner in each subunit (native α_2M) or by a disrupted corner (cleaved α_2M). The thiol ester in each subunit is represented by CO–S. The intermediate acyl group in 'nascent' α_2M is shown by *CO. Trypsin is shown as an open circle when bound to the 'bait' region of α_2M and as crosshatched circle when entrapped in α_2M. State I refers to the initial complex between α_2M and trypsin (binding mode I). State II indicates the cleavage of two 'bait' region sequences by one trypsin molecule. State III refers to 'nascent' α_2M. In this state two trypsin molecules are entrapped within one α_2M molecule due to the conformational change and the thiol esters have become cleaved resulting in appearance of free SH-groups. The acyl groups react rapidly with a fraction of the entrapped trypsin molecules to form covalent α_2M–trypsin complexes (state IV). Other nucleophiles that happen to be present can also react with the acyl groups (CO–X). State IV constitutes the final α_2M–trypsin complex and contains trypsin bound in modes II and III.

wise bound to α_2M by entrapment. During this binding the remaining 2 subunits of α_2M are also cleaved at the same Arg–Leu bonds in their 'bait' region. It is not clear which structures in α_2M constitute the final binding sites for trypsin or whether the two binding sites are identical.

The activated acyl-groups in 'nascent' α_2M–trypsin complex derived from the originally thiol esterified Glx-residues [13,14] may subsequently participate in 'acyl transfer' reactions [13] leading to formation of covalent α_2M–trypsin complexes. The fraction of acyl groups reacting with suitably oriented nucleophilic groups on the surface of trypsin may be expected to vary, dependent on the presence of other nucleophiles [16] besides water. Since α_2M–trypsin complexes formed at 2 mol trypsin/mol α_2M give rise to M_r 85 000 fragments on reducing SDS-PAGE [1,7–9] it appears that the larger part of trypsin bound covalently to α_2M only binds via 1 acyl group/trypsin molecule. However, trace amounts of components having higher M_r values are always found indicating that the activated acyl groups may cause a low degree of crosslinking of the M_r 85 000 fragments or that trypsin may bind covalently to 2 M_r 85 000 fragments.

Entrapped trypsin constitutes trypsin bound in mode II, while the fraction of trypsin that is furthermore bound covalently constitutes trypsin bound in mode III. In both of these modes the trypsin bound is protected against inhibition by STI.

The initial slope (fig.3 (×), (○)) corresponds to 1.07 mol trypsin bound/mol active trypsin. This shows that part of the inactive fraction constituting 24% of the total trypsin in the preparation of ^{125}I-labelled trypsin used here must have been bound to 'nascent' α_2M–trypsin complex in addition to the binding of active trypsin as anticipated [16]. When α_2M was incubated with trypsin at ratios $\lesssim$2 mol active trypsin/mol α_2M all the active trypsin bound to α_2M (maximally ~1.85–1.90 mol/mol α_2M for different preparations) was protected from inhibition by STI, regardless of whether 88% or 52% active trypsin was used. This result shows that binding of active trypsin resulting in protection of its active site takes place in preference to binding of inactive forms in binding modes II and III. The difference in the maximal amount of trypsin bound to α_2M (2.25 mol/mol, fig.3 (○)) and the amount protected (~1.85–1.90 mol/mol) may represent binding of not only inactive trypsin but also active trypsin in a mode where it can be inhibited by STI.

Fig.3 (×) shows that at 4.28 mol active trypsin added/mol α_2M, 3.54 mol trypsin is bound/mol α_2M. This result demonstrates that additional binding of trypsin (presumably up to 2 mol/mol α_2M) can take place in a mode different from modes II and III. This additional binding can be reversed by PTI (fig.3 (○)) indicating that the trypsin is bound via its active site. The binding sites on α_2M are most likely contributed by the 'bait' regions of 2 of the 4 subunits and are presumed to mediate binding analogous to binding mode I. Regarded in this way the 'bait' region constitutes a trypsin binding site that corresponds to the inhibitor site of 'classical' inhibitors such as PTI or STI [31,32].

Fig.3 (□) shows that the amount of trypsin bound to α_2M in the presence of 0.13 M CH_3NH_2 is only reduced by 2–5%, despite the simultaneous binding of 2.5–3.0 mol CH_3NH_2/mol 'nascent' α_2M trypsin complex under those conditions [16]. However, CH_3NH_2 clearly competes with trypsin for the covalent binding sites of α_2M since the amount of trypsin which remains bound, when examined after reduction and alkylation (conditions of fig.2) had decreased by ~50% (fig.3 (○), (■)). These results, and the earlier results showing that prior covalent binding of CH_3NH_2 to α_2M precludes subsequent binding of trypsin to α_2M [13,15] strongly support the model presented in fig.5 indicating that the covalent incorporation of trypsin into α_2M occurs on the originally thiol-esterified Glx-residues [13]. The maximal amount of trypsin bound covalently to α_2M is ~1.3 mol/mol α_2M at 2 mol trypsin/mol α_2M is compared with 1.85–1.90 mol trypsin protected/mol, here.

Evidently, covalent attachment is not a prerequisite for binding trypsin in a mode that confers protection of its active site against inhibition by STI. Furthermore, the titration curves for α_2M–trypsin complexes formed in the absence or the presence of 0.13 M CH_3NH_2 are identical (fig.4). This strongly indicates that the main factor responsible for the formation of protected α_2M–trypsin complexes is the conformational change in α_2M triggered by specific limited proteolysis in the 'bait' region. The simultaneous incorporation of 2.5–3.0 mol CH_3NH_2/mol α_2M only affects the distribution of trypsin bound in the protected modes II and III. The results shown in fig.4 also indicate that the kinetic properties of trypsin bound in these modes are very similar.

Acknowledgements

Technical assistance was given by Lene Kristensen, Mette Frandsen, Gitte B. Poulsen and Bente J. Christensen. Out-dated human plasma was obtained from The Blood Bank, Aarhus University Hospital through the courtesey of F. Kissmeyer-Nielsen, J. Jørgensen and S. Steenbjerg. This work was supported by the National Heart, Lung and Blood Institute, NIH (Bethesda MD) grant HL-16238.

References

[1] Harpel, P. C. (1973) J. Exp. Med. 138, 508–521.
[2] Barrett, A. J. and Starkey, P. M. (1973) Biochem. J. 133, 709–724.
[3] Werb, Z., Burleigh, M. C., Barrett, A. J. and Starkey, P. M. (1974) Biochem. J. 139, 359–368.
[4] Haverback, B. J., Dyce, B., Bundy, H. F., Wirtschafter, S. K. and Edmonson, J. (1962) J. Clin. Invest. 41, 972–980.
[5] Mehl, J. W., Connell, W. O. and Degroot, J. (1964) Science 145, 821–822.
[6] Ganrot, P. O. (1966) Clin. Chim. Acta 14, 493–501.
[7] Hall, P. K. and Roberts, R. C. (1978) Biochem. J. 171, 27–38.
[8] Swenson, R. P. and Howard, J. B. (1979) J. Biol. Chem. 254, 4452–4456.
[9] Barrett, A. J., Brown, M. A. and Sayers, C. A. (1979) Biochem. J. 181, 401–418.
[10] Sottrup-Jensen, L., Lønblad, P. B., Stepanik, T. M., Petersen, T. E., Magnusson, S. and Jörnvall, H. (1981) FEBS Lett. 127, 167–173.
[11] Topping, R. M. and Craven, A. H. (1979) Biochem. J. 177, 501–508.
[12] Ganrot, P. O. (1966) Acta Chem. Scand. 20, 2299–2300.
[13] Sottrup-Jensen, L., Petersen, T. E. and Magnusson, S. (1980) FEBS Lett. 121, 275–279.
[14] Sottrup-Jensen, L., Hansen, H. F., Mortensen, S. B., Petersen, T. E. and Magnusson, S. (1981) FEBS Lett. 123, 145–148.
[15] Swenson, R. P. and Howard, J. B. (1979) Proc. Natl. Acad. Sci. USA 76, 4313–4316.
[16] Sottrup-Jensen, L., Petersen, T. E. and Magnusson, S. (1981) FEBS Lett. 128, 123–126.
[17] Janatova, J., Lorenz, P. E., Schechter, A. N., Prahl, J. W. and Tack, B. F. (1980) Biochemistry 19, 4471–4478.
[18] Janatova, J., Tack, B. F. and Prahl, J. W. (1980) Biochemistry 19, 4479–4485.
[19] Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L. and Prahl, J. W. (1980) Proc. Natl. Acad. Sci. USA 77, 5764–5768.
[20] Pangburn, M. K. and Müller-Eberhard, H. J. (1980) J. Exp. Med. 152, 1103–1114.
[21] Sim, R. B., Twose, T. M., Paterson, D. S. and Sim, E. (1981) Biochem. J. 193, 115–127.
[22] Campbell, R. D., Dodds, A. W. and Porter, R. R. (1980) Biochem. J. 189, 67–80.
[23] Harpel, P. C. and Rosenberg, R. D. (1976) Progr. Thromb. 3, 145–189.
[24] Pochon, F., Amand, B., Lavalette, D. and Bieth, J. (1978) J. Biol. Chem. 253, 7496–7499.
[25] Salvesen, G. S. and Barrett, A. J. (1980) Biochem. J. 187, 695–701.
[26] Sayers, C. A. and Barrett, A. J. (1980) Biochem. J. 189, 255–261.
[27] Robinson, N. C., Tye, R. W., Neurath, H. and Walsh, K. A. (1971) Biochemistry 10, 2743–2747.
[28] Hunter, W. M. and Greenwood, F. C. (1962) Nature 194, 495.
[29] Dunn, J. T. and Spiro, R. G. (1967) J. Biol. Chem. 242, 5549–5555.
[30] Jones, J. M., Creeth, J. M. and Kekwick, R. A. (1972) Biochem. J. 127, 187–197.
[31] Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J. and Steigemann, W. (1974) J. Mol. Biol. 89, 73–101.
[32] Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H. and Blow, D. M. (1974) Biochemistry 13, 4212–4228.