Stoichiometry of reactions of α_2 -macroglobulin with trypsin and chymotrypsin

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The stoichiometry of the individual steps, i.e. polypeptide chain cleavage, hydrolysis of the putative thioester bond and conformational change, of the reaction between α_2 -macroglobulin and trypsin or chymotrypsin was analysed. The chain cleavage was monitored by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the thioester hydrolysis by both a spectroscopic and a fluorimetric technique and the conformational change by tryptophan fluorescence. A stoichiometry of close to 2:1 was obtained for all reactions. This finding indicates that the α_2 -macroglobulin half-molecule is an independent functional unit of the inhibitor, within which co-operativity between the two subunits may occur.

α_2 -Macroglobulin (α_2 M) is a high-relative-molecular-mass ($M_r \sim 725\,000$) tetrameric plasma proteinase inhibitor (Jones *et al.*, 1972; Hall & Roberts, 1978) with unique properties. It inhibits a wide variety of proteinases of different classes and with different specificities (Barrett & Starkey, 1973; Harpel, 1976). The binding of the proteinase is initiated by the enzyme cleaving α_2 M in a limited region of the polypeptide chain, the 'bait' region (Harpel, 1973; Barrett *et al.*, 1979; Swenson & Howard, 1979a; Sottrup-Jensen *et al.*, 1981b). This cleavage triggers a conformational change in the inhibitor (Barrett *et al.*, 1974, 1979; Björk & Fish, 1982; Gonias *et al.*, 1982; Branegård *et al.*, 1982) that results in non-covalent binding ('trapping') of the enzyme (Barrett & Starkey, 1973). The binding occurs in a manner that greatly decreases the activity of the proteinase against high- M_r substrates, as well as the reactivity of the enzyme with antibodies and protein proteinase inhibitors, but largely preserves the activity against low- M_r substrates (Barrett & Starkey, 1973; Harpel, 1976). The proteolytic cleavage also leads to the appearance of a free thiol group and of an activated glutamic acid residue (of unknown nature) in each α_2 M chain (Sottrup-Jensen *et al.*, 1980, 1981a; Howard, 1981; Salvesen *et al.*, 1981). The latter residue can react with amino groups on the pro-

teinase so that a proportion of the non-covalently bound enzyme molecules also become covalently linked to the inhibitor (Swenson & Howard, 1979b; Salvesen & Barrett, 1980; Salvesen *et al.*, 1981; Sottrup-Jensen *et al.*, 1981c; Wu *et al.*, 1981; Van Leuven *et al.*, 1981). These observations have led to the suggestion that the thiol group and the carboxy group of the glutamic acid residue form a labile thioester bond in native α_2 M (Sottrup-Jensen, *et al.*, 1980; Howard, 1981; Salvesen *et al.*, 1981). Certain primary amines inactivate human α_2 M (Steinbuch *et al.*, 1968; Harpel, 1976; Barrett *et al.*, 1979), presumably by nucleophilic cleavage of the thioester bonds (Swenson & Howard, 1979b, 1980; Sottrup-Jensen *et al.*, 1980; Howard, 1981). The loss of activity is accompanied by a conformational change similar to that induced by proteinases (Barrett *et al.*, 1979; Björk & Fish, 1982; Gonias *et al.*, 1982).

Most evidence indicates that each α_2 M molecule can bind two molecules of small proteinases, such as trypsin and chymotrypsin (Ganrot, 1966; Barrett *et al.*, 1979; Swenson & Howard, 1979a; Sottrup-Jensen *et al.*, 1980; Pochon *et al.*, 1981). However, the stoichiometry of the individual reactions of this inhibition, i.e. chain cleavage, putative thioester hydrolysis and conformational change, has not been equally well established. In the present paper we report a comprehensive analysis of these stoichiometries with α_2 M purified by two different procedures and with highly purified and carefully characterized enzyme preparations. We find that all reactions occur with a stoichiometry of close to 2:1, indicating that the

Abbreviations used: α_2 M, α_2 -macroglobulin; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; STI, soya-bean trypsin inhibitor; SDS, sodium dodecyl sulphate; DACM, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleinimide; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

α_2 M half-molecule constitutes an independent functional unit of the inhibitor.

Materials and methods

α_2 M was prepared from fresh-frozen human plasma by precipitation with poly(ethylene glycol) (Barrett *et al.*, 1979), zinc-chelate chromatography (Kurecki *et al.*, 1979; Sottrup-Jensen *et al.*, 1980) and gel chromatography on Sephacryl S-300 (Pharmacia, Uppsala, Sweden). An alternative purification procedure was chromatography on Cibacron Blue-Sepharose (Virca *et al.*, 1978), followed by gel chromatography on Sephacryl S-300. The purity and stability of both preparations was comparable with that of material used previously (Björk & Fish, 1982). The solvent for α_2 M in all experiments was 50mM-Hepes/0.1M-NaCl, pH 7.4.

Bovine trypsin (type III, twice-crystallized; EC 3.4.21.4) and STI (type IS) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. β -Trypsin was isolated from the commercial preparation by affinity chromatography on agarose-linked STI (Robinson *et al.*, 1971; Yung & Trowbridge, 1975). Active-site titrations of the purified enzyme were done with 4-nitrophenyl 4-guanidinobenzoate (Chase & Shaw, 1970), obtained from E. Merck, Darmstadt, Germany, and with 4-nitrophenyl *N*²-benzyloxycarbonyl-L-lysinate hydrochloride (Bender *et al.*, 1966) from EGA-Chemie, Steinheim, Germany. A typical preparation gave values of 0.87 ± 0.01 (s.d., $n = 5$) and 0.95 ± 0.02 ($n = 5$) mol of active sites/mol of protein with the two methods respectively. For each trypsin preparation the average of the two values obtained in this manner was used. α -Chymotrypsin A (thrice-crystallized; EC 3.4.21.1) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. The preparation had 0.92 ± 0.03 ($n = 6$) and 0.96 ± 0.04 ($n = 5$) mol of active sites/mol of protein by active-site titrations with *N-trans*-cinnamoylimidazole (EGA-Chemie) and 4-nitrophenyl acetate (Serva, Heidelberg, Germany) respectively (Schonbaum *et al.*, 1961; Kézdy & Kaiser, 1970). The average of these two values, 0.94 mol of active sites/mol of protein, was used throughout the work. The enzymes were dissolved in 1mM-HCl/10mM-CaCl₂.

The ability of α_2 M to inhibit the action of trypsin and chymotrypsin on high- M_r substrates was measured by a fibrin-gel assay. α_2 M was incubated at 25°C for 5 min with increasing amounts of enzyme; the final α_2 M concentration was $\sim 0.75 \mu\text{M}$. A portion (10 μl) of the incubation mixture was then applied on the surface of a 1-mm-thick fibrin gel (in 50mM-triethanolamine, pH 7.8), formed by clotting human fibrinogen (Kabi-

Vitrum, Stockholm, Sweden) with bovine thrombin (Carlström *et al.*, 1977). The gels were incubated at 37°C for 3 h or 20 h for analysis of trypsin and chymotrypsin activity respectively. The amount of free enzyme was calculated from the size of the lysis areas with the aid of a standard curve.

The binding of trypsin to α_2 M was also checked by an assay based on the ability of the inhibitor to protect the enzyme from inactivation by STI (Ganrot, 1966; Barrett & Starkey, 1973). α_2 M (final concn. $0.22 \mu\text{M}$) was incubated with increasing amounts of trypsin as in the fibrin-gel assay. STI was then added in a 5-fold molar ratio to the maximum amount of trypsin added, and remaining trypsin activity was measured after 1 min with the chromogenic substrate *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide (S-2222; KabiVitrum) at pH 7.4.

The stoichiometry of peptide-bond cleavage of α_2 M was investigated by incubating α_2 M, at a final concentration of $0.80 \mu\text{M}$, with enzyme for 5 min at 25°C. *N*- α -*p*-Tosyl-L-lysylchloromethane or *p*-tosyl-L-phenylalanylchloromethane (Sigma) was then added in experiments with trypsin and chymotrypsin respectively to a final concentration of 2 mM. The samples were denatured in 1% (w/v) SDS/0.3M- β -mercaptoethanol for 45 min at 37°C, and SDS/polyacrylamide-gel electrophoresis was done on 5% (w/v) gels (Weber & Osborn, 1969). After staining with Coomassie Brilliant Blue R-250, the gels were scanned at 600 nm in a Cary model-219 spectrophotometer (Varian Associates, Palo Alto, CA, U.S.A.). The extent of chain cleavage was estimated as the absorbance of the band migrating at a position corresponding to an M_r of about 85000 as a percentage of the total absorbance of all bands detected on the gel.

Two methods were used to analyse the stoichiometry of the appearance of thiol groups. In one of these, α_2 M (final concn. $0.13 \mu\text{M}$) was incubated with increasing amounts of enzyme for 5 min at 25°C. The fluorogenic thiol-group reagent DACM (Yamamoto *et al.*, 1977), obtained from Serva, was then added to a concentration of $10 \mu\text{M}$, and the fluorescence was measured in an SLM 4800S spectrofluorimeter (SLM Instruments, Urbana, IL, U.S.A.). The excitation and emission wavelength were 400 and 468 nm respectively, and the bandwidth of both channels was 4 nm. The fluorescence of each sample was measured exactly 150 min after addition of the reagent, since the fluorescence increases slowly with time, due to ring-opening of the fluorophore formed initially (Yamamoto *et al.*, 1977). The fluorescence of the reagent was subtracted.

The stoichiometry of thiol-group appearance was also analysed with Nbs₂ (Ellman, 1959). α_2 M (final concn. $2.2 \mu\text{M}$) was first incubated with tryp-

sin or chymotrypsin of increasing concentrations for 2 min at 25°C in the presence of 2 mM-EDTA. Nbs₂ (Fluka, Buchs, Switzerland) was then added to a final concentration of 100 μ M, and A_{412} was monitored in a Cary 219 spectrophotometer against a blank without α_2 M until constant (i.e. for 2–5 min). The number of thiol groups exposed was calculated from a molar absorption coefficient of the *p*-nitrothiophenolate anion of 13 600 M⁻¹·cm⁻¹ (Ellman, 1959).

The conformational change of α_2 M on reaction with proteinases was monitored by tryptophan fluorescence (Björk & Fish, 1982; Straight & McKee, 1982). A series of samples containing α_2 M at a constant final concentration (from 40 nM to 1.2 μ M in different experiments) and increasing amounts of trypsin or chymotrypsin, were incubated for 5 min at 25°C. The fluorescence of each sample was then measured in an SLM 4800S spectrofluorimeter with excitation and emission wavelengths of 279 and 330 nm respectively; the corresponding bandwidths were 2 and 8 nm. Large inner-filter corrections in the analyses of the samples containing 1.2 μ M- α_2 M were avoided by diluting these samples 10-fold before fluorescence measurements. The fluorescence increase caused by the enzymes were expressed as $(F_S - F_E - F_0)/F_0$, where F_S is the fluorescence of the sample, F_E the fluorescence of an enzyme solution of the same concentration as in the sample (corrected for the inner-filter effect due to the α_2 M in the sample) and F_0 the fluorescence of α_2 M alone at the same concentration as in the sample. Other corrections for inner-filter effects were insignificant.

Protein concentrations were obtained by absorption measurements at 280 nm. The specific absorption coefficients and M_r values used in the calculations have been reported previously (Björk & Fish, 1982).

Results and discussion

The following methods were selected for the analysis of the stoichiometry of the individual reactions involved in the inactivation of trypsin and chymotrypsin by α_2 M. The cleavage of the α_2 M polypeptide chain by the proteinases was monitored by SDS/polyacrylamide-gel electrophoresis, the appearance of thiol groups was analysed by both a spectroscopic and a fluorimetric technique, and the conformation change in the inhibitor was studied by tryptophan fluorescence. As a comparison with previous work, the stoichiometry of inhibition of the two proteinases was also analysed.

Representative plots of the results obtained by the different methods, all with α_2 M prepared by zinc-chelate chromatography, are presented in Figs. 1–5. Stoichiometries of the reactions were

Fig. 1. Stoichiometry of inhibition of proteinases by α_2 M, analysed by a fibrin-gel assay

○, Trypsin; □, chymotrypsin. The different slopes of the two lines representing the increase in free enzyme concentration after saturation of the inhibitor are due to slightly different α_2 M concentrations in the two analyses.

Fig. 2. Stoichiometry of peptide-bond cleavage of α_2 M, analysed by SDS/polyacrylamide-gel electrophoresis

○, Trypsin; □, chymotrypsin. The extent of cleavage was expressed as the intensity of the ~ 85000 - M_r band as a percentage of that of all bands on the gel. The apparent failure to achieve 100% cleavage (in spite of total disappearance of the band representing the intact α_2 M subunit) is due to the formation of some material migrating slower than the α_2 M subunit and to a small amount of secondary cleavage of the ~ 85000 - M_r fragments.

estimated from these and similar plots from the intersection of the two linear portions of the curves. Linear least-squares regression was used to fit the sloping part of each curve, and the numerical average was used to approximate the plateau region. Stoichiometries of 1.6–2.1 were obtained for the different reactions with both α_2 M preparations (Table 1); most values were between 1.7 and 1.9.

Fig. 3. Stoichiometry of thiol-group appearance in α_2M , analysed with DACM

○, Trypsin; □, chymotrypsin. An evaluation of the total number of liberated thiol groups is impossible in this analysis, since the quantum yield of the bound fluorophore is markedly dependent on the environment.

Fig. 4. Stoichiometry of thiol-group appearance in α_2M , analysed with Nbs_2

○, Trypsin; □, chymotrypsin. The number of thiol groups liberated per α_2M molecule by trypsin and chymotrypsin was 3.8 ± 0.1 (s.d.) and 3.7 ± 0.1 respectively.

The results for the stoichiometry of inactivation of the two proteinases are consistent with most previous reports (Ganrot, 1966; Barrett *et al.*, 1979; Swenson & Howard, 1979a; Sottrup-Jensen *et al.*, 1980; Pochon *et al.*, 1981); however, we obtained a higher value than that of 1.4 recently published for the inactivation of chymotrypsin (Howell *et al.*, 1983). A few analyses of the individual steps of the reactions of α_2M with the proteinases have been published, although a systematic investigation has not been made. Our data are in good agreement with the stoichiometry for

Fig. 5. Stoichiometry of conformational change of α_2M , analysed by tryptophan fluorescence

Reaction with trypsin: ○, α_2M concentration $1.22 \mu M$; ●, α_2M concentration $40 nM$.

the fluorescence increase caused by trypsin of about 2 measured by Straight & McKee (1982). In contrast, the stoichiometry of polypeptide chain cleavage obtained in this work is appreciably higher than the values of 1.3–1.4 and 1.5 reported for trypsin by Swenson & Howard (1979a) and Straight & McKee (1982) respectively. More significantly, by two methods we found a stoichiometry of thiol-group appearance for both enzymes of close to 2.0, i.e. a value markedly higher than that of about 1.0 suggested by Sottrup-Jensen *et al.* (1980) for the trypsin reaction. The reason for these discrepancies may be due partly to the use of highly purified and well-characterized enzyme preparations in the present work.

The variation in the observed stoichiometries from 1.6 to 2.1, and the average stoichiometry of slightly below 2.0, most likely is due largely to experimental error. However, those analyses in which the same reaction was studied at two markedly different protein concentrations, i.e. the appearance of thiol groups and the fluorescence increase (Table 1; Figs. 3–5), suggest that another factor may have contributed to the observed differences in stoichiometry. In these analyses, a lower apparent stoichiometry was obtained at the lower than at the higher protein concentration (1.6–1.7 as against 1.9–2.1). A possible explanation for this behaviour is that the rate of the non-covalent binding of an enzyme molecule (not necessarily the same as that which has cleaved the bait region) to the inhibitor is sufficiently slow in the more dilute solution that some binding sites created by the proteolytic cleavage may decay before binding a proteinase molecule. In the terminology of Barrett & Starkey (1973), perhaps the trap closes before an enzyme molecule is caught. Such non-productive

Table 1. *Stoichiometries of reactions of α_2 -macroglobulin with trypsin or chymotrypsin*
 α_2 M preparation methods were as follows: I, Zn^{2+} -Sephacrose; II, Blue Sepharose. Abbreviation used: ND, not determined.

Method of analysis	α_2 M concn. (μM)	α_2 M preparation method	Stoichiometry with:	
			Trypsin	Chymotrypsin
Inactivation of proteinase				
Fibrin-gel assay	0.7–0.83	I	1.8	1.7
	0.7–0.83	II	1.7	1.8
STI assay	0.22	I	1.9	ND
	0.22	II	1.9	ND
Cleavage of peptide bonds	0.80	I	1.8	1.9
Appearance of thiol groups				
DACM assay	0.13	I	1.6	1.7
	0.13	II	1.7	1.7
Nbs ₂ assay	2.20	I	1.9	2.1
Fluorescence	1.22	I	2.0	ND
	0.1	I	1.7	ND
	0.04	I	1.7	1.9
	0.1	II	1.8	1.7

activation of binding sites may, in fact, have occurred to a limited extent also at higher protein concentrations, leading to apparent non-integral stoichiometry. A similar proposal has been made by Howell *et al.* (1983) to explain the different stoichiometries observed with different enzymes. In view of this possibility, and of the similarity of the values in Table 1, we conclude that all the individual reactions of the binding of trypsin or chymotrypsin to α_2 M that were studied occur with a stoichiometry of 2:1.

The demonstration that all the steps of the inhibition are complete when two molecules of proteinase are bound to the inhibitor indicates that each α_2 M half-molecule is an independent functional unit of the inhibitor. This suggestion is consistent with previous observations (Jones *et al.*, 1972; Barrett *et al.*, 1979; Gonias & Pizzo, 1983). In particular, Gonias & Pizzo (1983) have isolated half-molecules with reasonably intact proteinase-binding ability by mere reduction and alkylation of the tetramer. Within each functional half of the intact inhibitor, the reaction sequence may be initiated by the first cleavage of a bait region, the second bait region being cleaved subsequently by the trapped proteinase or by free enzyme. Alternatively, the binding may be triggered by the second-bait-region cleavage. The ensuing reactions, i.e. the hydrolysis of the putative thioester bond and the conformational change creating the proteinase-binding site, most likely occur with co-operativity between the two subunits of each half-molecule. In contrast, the results provide no evidence for a co-operativity between the half-molecules, which thus appear to be functionally independent. These conclusions are consistent with the demonstration by Gonias & Pizzo (1983) that the two subunits of

each half-molecule strongly interact with each other, but that only weak interactions exist between the two half-molecules.

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