# Mechanism of $\alpha_2$-Macroglobulin–Proteinase Interactions. Studies with Trypsin and Plasmin†

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ABSTRACT: The time course of the interactions of $\alpha_2$-macroglobulin with trypsin and with plasmin was studied by measuring the generation of thiol groups, the concentration of $\alpha_2$-macroglobulin subunits cleaved at the bait regions, and the change in intrinsic protein fluorescence of $\alpha_2$-macroglobulin–enzyme reaction mixtures as functions of time. The interaction of $\alpha_2$-macroglobulin with trypsin was found to be very fast but could be studied in the presence of benzamidine, a rather strong competitive inhibitor of trypsin. The results obtained indicate that $\alpha_2$-macroglobulin–proteinase reactions, known to involve specific limited proteolysis of the bait regions, gross conformational changes, and cleavage of the internal $\beta$-cysteinyl-$\gamma$-glutamyl thiol esters of native $\alpha_2$-macroglobulin, may proceed via at least two different reaction pathways determined by the nature of or the concentration of the reacting proteinase. After initial cleavage of one bait region at high proteinase activity the next step presumably is a fast cleavage of a second bait region before any substantial rearrangements leading to generation of thiol groups and the final incorporation of the proteinase occur. At low proteinase activity no further bait region cleavages occur and only the two thiol groups of half of the $\alpha_2$-macroglobulin molecule are generated in the final 1:1 complex. Estimates of the initial association rate constants of the $\alpha_2$-macroglobulin–trypsin and the $\alpha_2$-macroglobulin–plasmin reactions were $2 \times 10^7$ $M^{-1}$ $s^{-1}$ and $5 \times 10^5$ $M^{-1}$ $s^{-1}$, respectively, apparently corresponding to the overall rate constants of the cleavage of one bait region in native $\alpha_2$-macroglobulin, that is, $k_c/K_m$ of that enzymatic step. After rearrangements within the complexes with generation of two of the possible four thiol groups, a further bait region cleavage step was more slow; the apparent rate constants were found to be $1.3 \times 10^6$ $M^{-1}$ $s^{-1}$ for trypsin and $3 \times 10^3$ $M^{-1}$ $s^{-1}$ for plasmin.

The tetrameric plasma glycoprotein $\alpha_2$-macroglobulin ($\alpha_2$M), mol wt 725 000, of known primary structure (Sottrup-Jensen et al., 1983a), interacts with and influences the activity of most endopeptidases including aspartic, cysteine, and serine proteinases and metalloproteinases. The interaction of $\alpha_2$-macroglobulin and proteinases requires catalytically active enzymes, and complex formation is initiated by specific limited proteolysis in the so-called bait region (Harpel, 1973; Barrett & Starkey, 1973), located as residues 681–711 of each subunit (Sottrup-Jensen et al., 1983a).

Following this cleavage $\alpha_2$-macroglobulin undergoes a conformational change, which results in the formation of a binding site (or sites) for proteinases and the appearance of a recognition site for receptors found on different cells (Debanne et al., 1975; Van Leuven et al., 1979; Kaplan & Nielsen, 1979; Willingham et al., 1979; Gliemann et al., 1983). Furthermore, sulfhydryl groups (maximally 4 mol/mol of $\alpha_2$M) appear as a result of cleavage of the internal $\beta$-cysteinyl-$\gamma$-glutamyl thiol esters, formed by Cys-949 and Glx-952 (Sottrup-Jensen et al., 1980, 1983a; Howard, 1981; Salvesen et al., 1981).

Only a few studies dealing with the rates of interactions of $\alpha_2$-macroglobulin and proteinases have been presented, but the overall rates seem to differ by several orders of magnitude (Iwamoto & Abiko, 1970; Wuepper & Cochrane, 1972; Downing et al., 1978; Salvesen et al., 1981; Straight & McKee, 1982; Howell et al., 1983). The objective of the present study was to investigate the kinetics of the interactions of $\alpha_2$-macroglobulin with trypsin (EC 3.4.21.4) and with plasmin (EC 3.4.21.7) in order to possibly obtain information for a more detailed description of these reactions. Part of the results have been reported in brief earlier (Sottrup-Jensen et al., 1983b).

## Experimental Procedures

*Materials.* Human $\alpha_2$-macroglobulin was prepared by $Zn^{2+}$ affinity chromatography as described by Sottrup-Jensen et al. (1980). Its concentration was determined from the absorbance measured at 280 nm with $E^{1\%}_{1cm} = 9.1$ (Dunn & Spiro, 1967) and the mol wt 725 000 (Jones et al., 1972). Aliquots stored at −20 °C in 0.1 M $Na_2HPO_4/NaH_2PO_4$ buffer, pH 8.0, were thawed, filtered through 0.47-$\mu$m membrane filters, and kept at 4 °C for no more than 24 h before use. $^{125}$I-Labeled $\alpha_2$-macroglobulin, $^{125}$I-labeled plasminogen, and $^{125}$I-labeled trypsin were prepared by the chloramine T procedure using $Na^{125}I$ from Amersham, Buckinghamshire, U.K. (Hunter & Greenwood, 1962).

Bovine trypsin was a gift from Novo, Copenhagen, Denmark (Trypure). A stock solution in 1 mM HCl was kept at −20 °C in aliquots. Its active-site concentration was 176 $\mu$M (82% active) as determined by 4-nitrophenyl 4-guanidinobenzoate titration (Chase & Shaw, 1969). Trypsin used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis experiments was from Boehringer, Mannheim, West Germany.

Human plasminogen was purified essentially as described by Deutsch & Mertz (1970). Human plasmin, used in thiol group generation experiments, was prepared from plasminogen by activation on a column of urokinase-substituted Sepharose 4B (Christensen, 1975). Soybean trypsin inhibitor was from BDH, Poole, U.K. Benzamidine, 5,5′-dithiobis(2-nitrobenzoic acid), and other chemicals were analytical grade from Merck, Darmstadt, FRG, or from Fluka, Buchs, Switzerland.

*Methods.* The time course of thiol group generation in the reactions of $\alpha_2$-macroglobulin with the enzymes was followed by measuring the change in absorbance at 412 nm in the presence of a large excess of 5,5′-dithiobis(2-nitrobenzoic acid) at 25 °C in a Beckman 35 double-beam spectrophotometer. To each of two semimicrocuvettes (light path length 10 mm,

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0006-2960/84/0423-6619$01.50/0 

dimensions 10 mm × 4 mm × 30 mm) were added 5,5′-dithiobis(2-nitrobenzoic acid) (final concentration 1 mM), $\alpha_2$-macroglobulin (final concentration 2.5 $\mu$M), and the desired amount of 0.1 M $NaH_2PO_4/Na_2HPO_4$ buffer, pH 7.6, and the difference absorbance was set to zero. After addition of buffer solution to the reference cuvette, the same volume of enzyme solution (trypsin–benzamidine mixture or plasmin) was added to the sample cuvette and the reaction was followed for 30–120 min. In the absence of $\alpha_2$-macroglobulin no changes in difference absorbance were seen. The concentration of generated thiol groups at time $t$ was calculated by using $\epsilon$ = 13 600 (Habeeb, 1972).

To follow the time course of bait region cleavage in the reaction of $^{125}$I-labeled $\alpha_2$-macroglobulin with benzamidine-inhibited trypsin, 30-$\mu$L samples from reaction mixtures containing benzamidine (125 mM), $^{125}$I-labeled $\alpha_2$-macroglobulin (2.5 $\mu$M), and trypsin (10 $\mu$M) were taken at various times after initiation of the reaction and mixed with 30 $\mu$L of a solution of sodium dodecyl sulfate (2% w/v), urea (4 M), and dithioerythritol (20 mM), kept in a boiling water bath. After these mixtures were incubated for 5 min at 100 °C, sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 7.5% slab gels.

Samples (10 $\mu$L) containing approximately 60 000 cpm of $^{125}$I-labeled $\alpha_2$-macroglobulin were applied to each lane. After the gels were stained with Coomassie brilliant blue and destained in 10% acetic acid, the bands were cut out and counted for 10 min in an LKB Ultrogamma counter. The recovery of radioactivity was 85–95%.

Similar experiments were performed with plasmin and $\alpha_2$-macroglobulin. $^{125}$I-Labeled $\alpha_2$-macroglobulin (2.5 $\mu$M) and plasmin (molar ratio 1–3; total volume 100 $\mu$L) were incubated 1 or 60 min. Samples (30 $\mu$L) were removed and treated as described above.

The amount of trypsin bound covalently to $\alpha_2$-macroglobulin was determined from experiments in which unlabeled $\alpha_2$-macroglobulin (2.5 $\mu$M) and $^{125}$I-labeled trypsin (10 $\mu$M; benzamidine, 125 mM) were incubated, and 10-$\mu$L samples containing approximately 100 000 cpm of $^{125}$I-labeled trypsin were taken and applied to nonreducing sodium dodecyl sulfate gel electrophoresis. The radioactivity associated with the mol wt 360 000 half-molecules of $\alpha_2$-macroglobulin was determined.

Binding of plasmin to $\alpha_2$-macroglobulin was determined essentially as described by Sottrup-Jensen et al. (1981b). Samples (1 mL) of $\alpha_2$-macroglobulin (2.5 $\mu$M) and $^{125}$I-labeled plasmin (molar ratio 0.25–4.0) were incubated at 22–23 °C for 90 min and then applied to gel chromatography on Sephacryl S-300.

The time course of the change in intrinsic fluorescence of $\alpha_2$-macroglobulin after addition of plasmin was followed by use of an SLM 4800S fluorescence spectrophotometer (SLM Instruments, Urbana, IL). Excitation was at 280 nm, and fluorescence emission was measured at 340 nm. The excitation and emission bandwidths were 2 and 16 nm, respectively. The experiments were done with equal concentrations of $\alpha_2$-macroglobulin and plasmin in the range 0.1–1.0 $\mu$M.

## Results and Discussion

*Interaction of $\alpha_2$-Macroglobulin with Trypsin.* Although $\alpha_2$-macroglobulin contains four subunits with identical primary structure (Sottrup-Jensen et al., 1983a), it binds only two molecules of trypsin tightly; $K_D < 10^{-13}$ M (Christensen & Sottrup-Jensen, 1983). At concentrations of trypsin less than that of $\alpha_2$-macroglobulin ([$\alpha_2$M] = 1–10 $\mu$M), essentially only 1:1 trypsin–$\alpha_2$-macroglobulin complexes are formed. In such

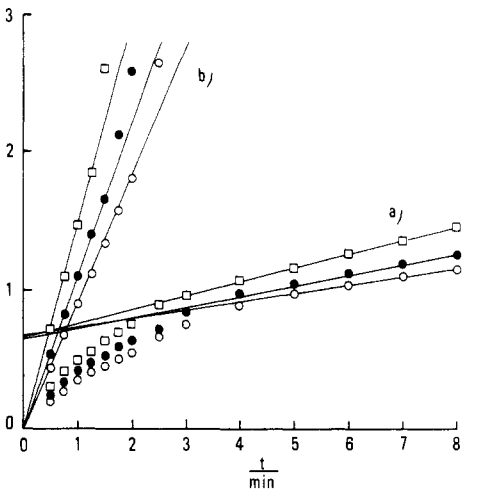


FIGURE 1: Time course of liberation of thiol groups in the reaction of $\alpha_2$-macroglobulin with trypsin at a number of very low concentrations of free, active trypsin obtained in the presence of benzamidine. In (a) $-\ln(1 - [SH]/[SH_{tot}])$ and in (b) $-\ln[1 - [SH]/(0.5[SH_{tot}])]$ are plotted against time. [SH] is the concentration of thiol groups obtained at time $t$ and $[SH_{tot}]$ is that at $t = \infty$. The total concentrations of trypsin were 9.5 (□), 7.1 (●), and 5.7 $\mu$M (○) and the concentration of benzamidine was 94 mM, so that the concentrations of free, active trypsin were $1.2 \times 10^{-9}$ (□), $9.1 \times 10^{-10}$ (●), and $7.3 \times 10^{-10}$ M (○). The concentration of $\alpha_2$-macroglobulin was 2.5 $\mu$M in all cases.

1:1 complexes the thiol esters from each of the four subunits of $\alpha_2$-macroglobulin are cleaved (Sottrup-Jensen et al., 1980, 1981b; Christensen & Sottrup-Jensen, 1983), and the change from the electrophoretically slow to the fast form of $\alpha_2$-macroglobulin (Sottrup-Jensen et al., 1983b) is complete, as also seen from the results presented by Van Leuven et al. (1981). The stoichiometry of thiol groups generated and trypsin molecules bound is thus 4:1 for 1:1 trypsin–$\alpha_2$-macroglobulin complexes and 2:1 for 2:1 complexes as discussed earlier (Christensen & Sottrup-Jensen, 1983). Here the time course of the interaction of trypsin and $\alpha_2$-macroglobulin was found to be too fast to study with conventional techniques. At concentrations of trypsin 0.1–10 times that of $\alpha_2$-macroglobulin ([$\alpha_2$M] = 2.5 $\mu$M) approximately 90–95% of the thiol groups finally generated had appeared when measuring was initiated, that is, in less than 10–15 s, while the remaining 5–10% appeared within 30–100 s. Therefore, the interaction of $\alpha_2$-macroglobulin with trypsin was investigated in the presence of benzamidine, a strong competitive inhibitor of trypsin (using Cbz-Val-Gly-Arg-*p*-nitroanilide as substrate, we found $K_i$ = 12 $\mu$M). When total concentrations of trypsin (much) greater than that of $\alpha_2$-macroglobulin are used in the presence of benzamidine ($10^3$–$10^4$ times $K_i$), not only a very low but also a nearly constant concentration of free, active trypsin will be present during the reaction. The generation of thiol groups under such conditions was slow, but the final amount was the same as in the absence of benzamidine. Figure 1 shows representative plots of the generation of thiol groups during the reaction of trypsin and $\alpha_2$-macroglobulin in the presence of 94 mM benzamidine. In these plots $-\ln(1 - [SH]/[SH_{tot}])$ is plotted against time $t$. [SH] is thiol groups formed at time $t$, and $[SH_{tot}]$ is thiol groups finally formed. If only one (pseudo) first-order process occurred, these plots would have been rectilinear. The curves of Figure 1, however, are all biphasic, and in all cases extrapolation of the final rectilinear portions of the curves to zero time gives $-\ln(1 - [SH]/[SH_{tot}])_{t=0} = \ln 2$; that is, $[SH] = 0.5[SH_{tot}]$. Presumably a two-step reaction in which half of the thiol groups are generated in a fast reaction step and the other half in a slower reaction step is revealed. The slopes of the lines

Table I: Values of Rate Constants $k_1$ and $k_2$ in the Reaction between $\alpha_2$-Macroglobulin (2.5 $\mu$M) and Trypsin in the Presence of Trypsin Inhibitors[a]

| inhibitor | total concn of inhibitor (mM) | total concn of trypsin ($\mu$M) | $[T_a]^b$ (nM) | $k_1$ ($min^{-1}$) | $k_2$ ($min^{-1}$) |
|---|---|---|---|---|---|
| benzamidine | 25 | 14.4 | 6.9 | (too fast) | 0.54 |
| benzamidine | 25 | 9.5 | 4.6 | (too fast) | 0.36 |
| benzamidine | 25 | 7.1 | 3.4 | (too fast) | 0.26 |
| benzamidine | 25 | 5.7 | 2.7 | (too fast) | 0.21 |
| benzamidine | 63 | 14.4 | 2.7 | (too fast) | 0.22 |
| benzamidine | 63 | 9.5 | 1.8 | ~2.2 | 0.14 |
| benzamidine | 94 | 14.4 | 1.8 | ~2.5 | 0.15 |
| benzamidine | 63 | 7.1 | 1.35 | 1.7 | 0.11 |
| benzamidine | 94 | 9.5 | 1.21 | 1.5 | 0.095 |
| benzamidine | 63 | 5.7 | 1.09 | 1.3 | 0.085 |
| benzamidine | 94 | 7.1 | 0.91 | 1.1 | 0.070 |
| benzamidine | 126 | 9.5 | 0.91 | 1.1 | 0.067 |
| benzamidine | 126 | 7.1 | 0.68 | 0.85 | 0.065 |
| benzamidine | 126 | 5.7 | 0.54 | 0.65 | 0.050 |
| STI[c] | 0.0214 | 9.5 | 0.4 | 0.5 | ~0.032 |
| STI | 0.0286 | 9.5 | 0.25 | 0.3 | (too slow) |

[a] The rate constants $k_1$ and $k_2$ were for the two reaction steps in each of which two thiol groups were generated. The values of $k_1$ and $k_2$ were obtained as illustrated in Figure 1. [b] Concentration of free, active trypsin. [c] Soybean trypsin inhibitor.

in Figure 1a, in that case, represent the rate constant of the slower step, $k_2$, the generation of the second pair of thiol groups, at the concentration of trypsin present.

In Figure 1b the time course of the generation of the first pair of thiol groups is illustrated; $-\ln [1 - [SH]/(0.5[SH_{tot}])]$ is plotted against time. The curves are rectilinear initially as would be expected if indeed half of the thiol groups are generated in a faster reaction step separated from the generation of the other half. As long as the slower reaction step has not contributed significantly, the curves of Figure 1b should be rectilinear and their slopes represent the rate constant of the faster reaction step, $k_1$, at the actual concentration of trypsin. A number of experiments using various trypsin and benzamidine concentrations and an $\alpha_2$-macroglobulin concentration of 2.5 $\mu$M all gave results similar to those illustrated in Figure 1. They are summarized in Table I. The same picture was seen when soybean trypsin inhibitor was used to obtain a very low, active concentration of trypsin. No obvious relation is seen between the values of $k_1$ or $k_2$ and the *total* concentration of trypsin, but as seen from Figure 2 the values are proportional to the concentration of free, active trypsin $[T_a]$ calculated by using the mass action law and the $K_i$ values of trypsin–benzamidine and trypsin–soybean trypsin inhibitor complexes. From the slopes of the lines in Figure 2 two second-order rate constants representing the faster and the slower steps of generation of one pair of thiol groups were determined to be $k_{1(T)} = 2 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{2(T)} = 1.3 \times 10^6\ M^{-1}\ s^{-1}$, respectively, corresponding to eq 1:

$$\alpha_2M \xrightarrow[1]{\text{fast}} \alpha_2M(2SH) \xrightarrow[2]{\text{slow}} \alpha_2M(4SH) \qquad (1)$$

It is generally accepted that the interaction of $\alpha_2$-macroglobulin with a proteinase is initiated by specific limited proteolysis of $\alpha_2$-macroglobulin catalyzed by the reacting proteinase. In that case reaction 1 of eq 1 is composed of at least two steps, the initial cleavage of one of the $\alpha_2$-macroglobulin subunits at the bait region (a bait region cleavage step) and the actual generation of the first pair of thiol groups (a thiol group generating step). Whether reaction 2 of eq 1 contains a bait region cleavage step or not is not known. Alternative reaction pathways are illustrated in eq 2A and 2B:

$$\alpha_2M \underset{a}{\overset{1}{\rightleftarrows}} \underset{b}{} \alpha_2M(I)(2SH) \underset{c}{\overset{2}{\rightleftarrows}} \underset{d}{} \alpha_2M(II)(4SH) \quad (2A)$$

$$\alpha_2M \underset{a}{\overset{1}{\rightleftarrows}} \underset{e}{} \alpha_2M(II)(2SH) \underset{d}{\overset{2}{\rightleftarrows}} \underset{f}{} \alpha_2M(II)(4SH) \quad (2B)$$

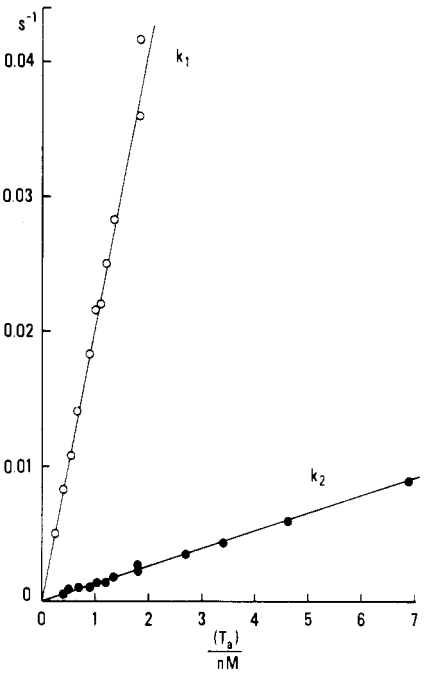


FIGURE 2: Dependence of rate constants $k_1$ and $k_2$ on the concentration of free, active trypsin $[T_a]$. The rate constants, $k_1$ and $k_2$, are the pseudo-first-order rate constants obtained as illustrated in Figure 1 of a fast ($k_1$) and a slow ($k_2$) generation of thiol groups in the reaction of $\alpha_2$-macroglobulin with trypsin at a number of very low concentrations of free, active trypsin, $[T_a]$. The values of $k_1$ (○) and $k_2$ (●) ($s^{-1}$) are plotted against $[T_a]$ (nM).

where steps a, c, and e are bait region cleavage steps and steps b, f, and d are thiol group generating steps.

The rates of the bait region cleavage steps catalyzed by trypsin must be proportional to the concentration of active trypsin. At the same total concentration of trypsin the rates are $10^3$–$10^5$ times as small in the presence of benzamidine as in its absence. It seems unlikely that the thiol generating steps should require free, active trypsin. They are probably rearrangement steps and therefore are kinetically first order with respect to the concentration of the intermediate that precedes the generation of the thiol groups in question.

At very low concentrations of free, active trypsin two thiol group generation reactions, both of which were trypsin dependent, could be distinguished. In these experimental conditions the rate-determining steps of both reaction 1 and reaction 2 of eq 1 are thus trypsin dependent, which indicates

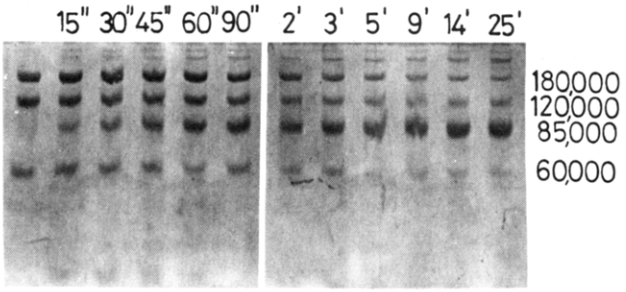


FIGURE 3: Time course of bait region cleavage in the reaction of $^{125}$I-labeled $\alpha_2$-macroglobulin (2.5 $\mu$M) with benzamidine (125 mM) inhibited trypsin (10 $\mu$M). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 7.5% slab gels after mixing of samples with sodium dodecyl sulfate (2% w/v), urea (4 M), and dithioerythritol (20 mM) (5 min, 100 °C).

that the sequence of events is that corresponding to eq 2A and that $k_{1(T)} = 2 \times 10^7$ M$^{-1}$ s$^{-1}$ is the second-order rate constant of the initial bait region cleavage step and $k_{2(T)} = 1.3 \times 10^6$ M$^{-1}$ s$^{-1}$ is that of a later bait region cleavage step, which comes after the generation of the first pair of thiol groups in the reaction sequence.

In the experiments in the absence of benzamidine the concentration of active trypsin is $10^3$–$10^5$ times as great as in its presence, and the rates of the bait region cleavage steps therefore are also $10^3$–$10^5$ times as great. A total of 90–95% of the thiol groups were generated before measuring was initiated, corresponding to 100% of the first pair plus 80–90% of the second pair of thiol groups.

The time courses of thiol group generation at high trypsin concentrations, although fast, are far too slow to be determined by $k_{2(T)}$, as can be seen from the following example: At 10 $\mu$M trypsin the half-time of the slower reaction would be of the order of magnitude (ln 2)/(1.3 × 10) = 0.054 s, and therefore all thiol groups would have appeared in less than 3–4 s (allowing 1% experimental error), not in approximately 30 s as seen experimentally. Under these conditions presumably the bait region cleavage step(s) becomes (become) fast and later steps determine the rate of thiol group generation.

The assignment of $k_{1(T)}$ to the initial bait region cleavage step was substantiated when the time course of bait region cleavages of $^{125}$I-labeled $\alpha_2$-macroglobulin (2.5 $\mu$M) was investigated at a total trypsin concentration of 10 $\mu$M in the presence of 125 mM benzamidine, and reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to separate the fragments of $\alpha_2$-macroglobulin formed. The results are shown in Figures 3 and 4. The cleavage of the bait region converts the mol wt 180 000 subunit into two mol wt 85 000 fragments after reduction (Harpel, 1973). In the experimental condition used to stop the protein–protein reaction here (boiling for 5 min) a large amount of nonproteolytic cleavage of intact mol wt 180 000 subunits is known to occur at the N-terminal side of the thiol-esterified Glx residue, so that one mol wt 60 000 fragment and one mol wt 120 000 fragment are generated (Barrett & Starkey, 1973; Harpel, 1973; Howard, 1981). The sum of the radioactivity of the mol wt 180 000, 120 000, and 60 000 fragments thus represents $\alpha_2$-macroglobulin subunits *not cleaved* at the bait region, whereas that of the mol wt 85 000 fragments represents the amount of subunits cleaved there. The fragments having molecular weights larger than 180 000 never contained more than 5% of the total radioactivity and were not included in these calculations. Total proteolysis in one bait region should take place in approximately 60 s under the conditions employed if indeed $k_{1(T)}$ is the second-order rate constant of the initial bait region cleavage step. Figure 4 shows that 25–30% of the

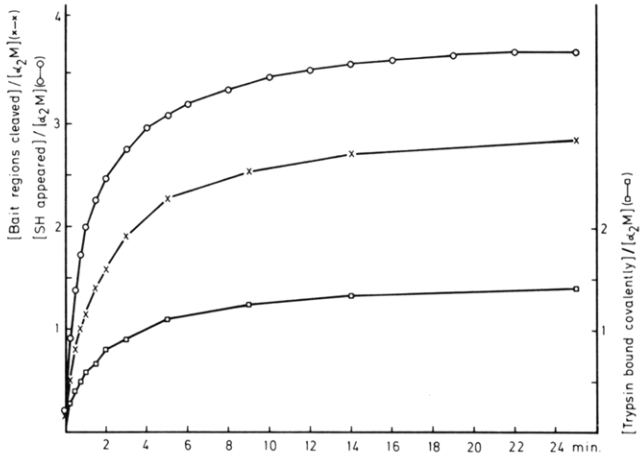


FIGURE 4: Correlation between generation of thiol groups (O), bait region cleavages (×), and covalent $\alpha_2$-macroglobulin–trypsin complex formation (□). Covalent binding data were obtained from the amount of $^{125}$I-labeled trypsin associated with mol wt 360 000 half-molecules of unlabeled $\alpha_2$-macroglobulin in sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the absence of dithioerythritol.

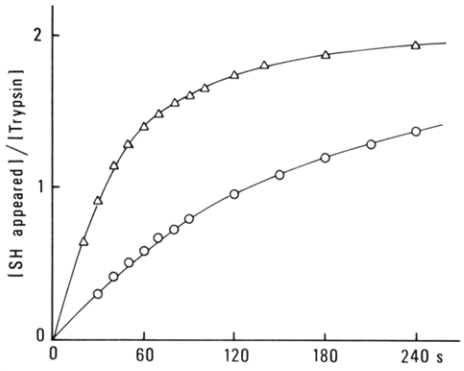


FIGURE 5: Time courses of generation of thiol groups in the interaction of $\alpha_2$-macroglobulin with trypsin at a very low concentration of free, active trypsin and a total concentration of trypsin 0.5 times that of $\alpha_2$-macroglobulin (2.0 $\mu$M) in the presence of 24 (Δ) and 48 mM (O) benzamidine. The lines show the theoretical time courses of the reaction using the rate equation $d[2SH]/dt = k_{1(T)}[T_a][\alpha_2M]$, where $[T_a]$ is the actual concentration of free, active trypsin at time $t$, $[\alpha_2M]$ is that of residual native $\alpha_2$-macroglobulin, and $k_{1(T)} = 2 \times 10^7$ M$^{-1}$ s$^{-1}$.

total radioactivity has appeared in mol wt 85 000 fragments after 60 s. As is further expected, the ratio [SH generated]/[bait regions cleaved] is 2 for the faster reaction step (initial part of the curve, Figure 4). In contrast to this, the slower generation of the remaining pair of thiol groups (complete in 20–25 min, here in the presence of benzamidine) is not clearly correlated with the average cleavage of two bait regions. Some second bait region cleavages seem to be inefficient in triggering the generation of thiol groups. Statistically one-third of these cleavages belong to that half of the $\alpha_2$-macroglobulin molecule that has already reacted (to yield 2 SH groups), and if their cleavage is inefficient, an average of 2.5 and not only 2 bait regions per $\alpha_2$-macroglobulin molecule have to be cleaved before all of the thiol groups can appear. The number actually seen is approximately 2.8 (70%) (Figure 4). This would explain the results if, as assumed here, the rates of generation of thiol groups at a low concentration of active trypsin are the same as that of a triggering bait region cleavage step.

The results reported above were obtained at total concentrations of trypsin greater than 2 times that of $\alpha_2$-macroglobulin. Figure 5 shows two examples of time courses of the generation of thiol groups in the interaction of trypsin and

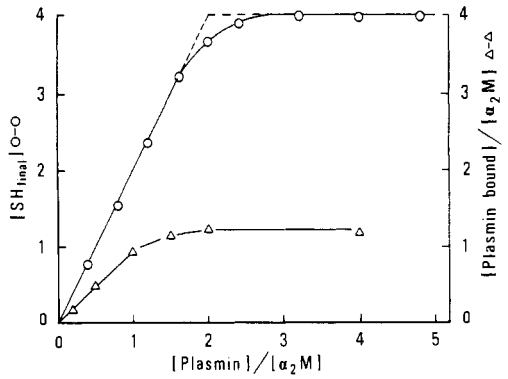


FIGURE 6: Final generation of thiol groups in the interaction of $\alpha_2$-macroglobulin (2.0 $\mu$M) with various concentrations of plasmin (0–5 times [$\alpha_2$M]) (O) and the molar amount of $\alpha_2$-macroglobulin-bound $^{125}$I-labeled plasmin obtained after gel filtration (Δ).

$\alpha_2$-macroglobulin at a very low, active concentration of trypsin and a total concentration of trypsin 0.5 times that of $\alpha_2$-macroglobulin. In such conditions only two thiol groups per trypsin are generated during the reactions. The time courses of the reactions correspond well with the theoretical ones for a process progressing as a second-order reaction with respect to the concentrations used of inhibited trypsin and of $\alpha_2$-macroglobulin and $k_{1(T)} = 2 \times 10^7$ M$^{-1}$ s$^{-1}$ (Figure 5). Again the rate-determining step is presumably the cleavage of one bait region of $\alpha_2$-macroglobulin with concomitant release of one pair of thiol groups and incorporation of trypsin. When a 1:1 trypsin–$\alpha_2$-macroglobulin complex is formed at a very low concentration of free, active trypsin, no release of the second pair of thiol groups is seen. At relatively high trypsin concentrations an average of two bait regions are cleaved (Swenson & Howard, 1979) before trypsin is inhibited so that no further cleavages take place, and in this case all four thiol groups are generated (Sottrup-Jensen et al., 1980; Christensen & Sottrup-Jensen, 1983). As judged from the last 5–10% of the thiol group generation curves obtained at high concentrations (micromolar range) of trypsin, a rate constant of the order of magnitude $k_{SH} \approx 0.2$ s$^{-1}$ determines that step, indicating that the rate of a second bait region cleavage step (rate proportional to $k_{2(T)}[T_a]$) and the rate of the rearrangements leading to thiol group generation (one pair) (rate proportional to $k_{SH}$) may become equal at a concentration of trypsin, $[T_a]$, approximately equal to $k_{SH}/k_{2(T)} \simeq 0.1$ $\mu$M. The change of reaction pathway then should occur in the range 0.02–0.5 M concentration of trypsin. The extent of formation of covalent trypsin–$\alpha_2$-macroglobulin complexes seems to be the same (maximally 60–70%), regardless of whether the complexes have been formed at micromolar concentrations of trypsin (Sottrup-Jensen et al., 1981b) or as here at very low concentrations (Figure 4).

*Interaction of $\alpha_2$-Macroglobulin with Plasmin.* Figure 6 shows the concentration of thiol groups generated after 2 h from $\alpha_2$-macroglobulin–plasmin complexes in reaction mixtures containing different concentrations of plasmin and a fixed concentration of $\alpha_2$-macroglobulin. These results are in striking contrast to those obtained for the interaction of trypsin with $\alpha_2$-macroglobulin in similar experimental conditions but are equivalent to those obtained at very low concentrations of free, active trypsin. The stoichiometry of thiol groups generated and plasmin molecules bound is 2:1 for 1:1 as well as for 2:1 plasmin–$\alpha_2$-macroglobulin complexes. One of the plasmin molecules of the possible 2:1 complexes is not bound as tightly as the other one, since after gel filtration in a nondenaturing solvent a maximum of 1.2 plasmin molecules

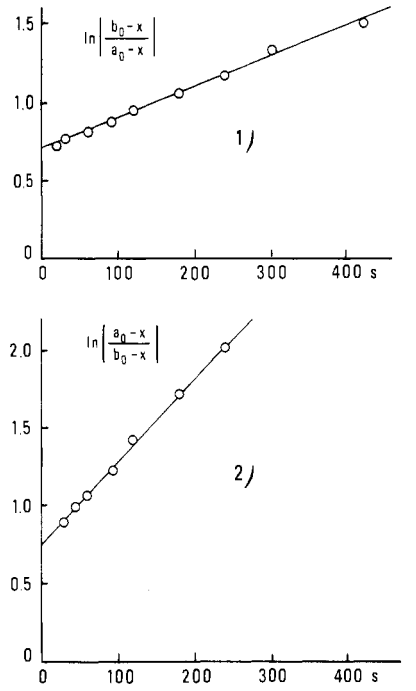


FIGURE 7: Kinetics of generation of the second pair of thiol groups in the interaction of $\alpha_2$-macroglobulin with plasmin at concentrations of plasmin greater than that of $\alpha_2$-macroglobulin. [Complete formation of a 1:1 complex generating one pair of thiol groups per $\alpha_2$-macroglobulin in a rather fast reaction step apparently is obtained before measuring is initiated (approximately 10 s after mixing).] $a_0$ is the concentration of residual, free plasmin after complete formation of the 1:1 $\alpha_2$-macroglobulin–plasmin complex. $b_0$ is the total concentration of $\alpha_2$-macroglobulin and is taken to be the initial concentration of the 1:1 complex for the second reaction step. $x$ is the concentration of the second pair of thiol groups generated at time $t$ and is considered to be equal to the concentration of the 1:2 $\alpha_2$-macroglobulin–plasmin complex at time $t$. $\ln [(a_0 - x)/(b_0 - x)]$ or $\ln [(b_0 - x)/(a_0 - x)]$ is plotted against time, $t$ (s). The rate equation for a second-order process A + B $\xrightarrow{k}$ X may be expressed as follows: $\ln [(a_0 - x)/(b_0 - x)] = (a_0 - b_0)kt + \ln (a_0/b_0)$. According to this equation $k$ values obtained from the two curves shown are as follows: (curve 1) $k = 2.1 \times 10^3$ M$^{-1}$ s$^{-1}$, where $a_0 = 1.0$ $\mu$M, $b_0 = 1.9$ $\mu$M, and the slope is $1.9 \times 10^{-3}$ s$^{-1}$; (curve 2) $k = 2.4 \times 10^3$ M$^{-1}$ s$^{-1}$, where $a_0 = 4.1$ $\mu$M, $b_0 = 1.9$ $\mu$M, and the slope is $5.3 \times 10^{-3}$ s$^{-1}$.

was found to be bound to one $\alpha_2$-macroglobulin molecule, but 2:1 complexes presumably *are* formed (Figure 6). In the molar range 0–1 plasmin per $\alpha_2$-macroglobulin two thiol groups per plasmin are generated, and the plasmin bound in the resultant 1:1 complex does *not* attack the residual free $\alpha_2$-macroglobulin. If that was so, all of the thiol groups should appear no matter what was the concentration of plasmin. The same is true in the concentration range 1–2 plasmins per $\alpha_2$-macroglobulin. If the second plasmin is free, all of the thiol groups and not only an amount stoichiometrically related to that of plasmin should appear finally. The thiol group generation pattern thus strongly indicates that not only 1:1 but also 2:1 plasmin–$\alpha_2$-macroglobulin complexes do form.

The time course of the interaction of plasmin with $\alpha_2$-macroglobulin was investigated at concentrations of plasmin 0.1–4 times that of $\alpha_2$-macroglobulin (1.9 $\mu$M) in the absence of other plasmin inhibitors. At concentrations of plasmin 0.1–1 times that of $\alpha_2$-macroglobulin, approximately 80–90% of the two thiol groups per plasmin added that were finally generated had appeared when measuring was initiated, that is, after approximately 10 s of reaction time. At concentrations of plasmin greater than that of $\alpha_2$-macroglobulin approximately one pair of thiol groups had appeared initially and then the second pair (or the fraction of it equivalent to the excess concentration of plasmin) was generated in a rather slow

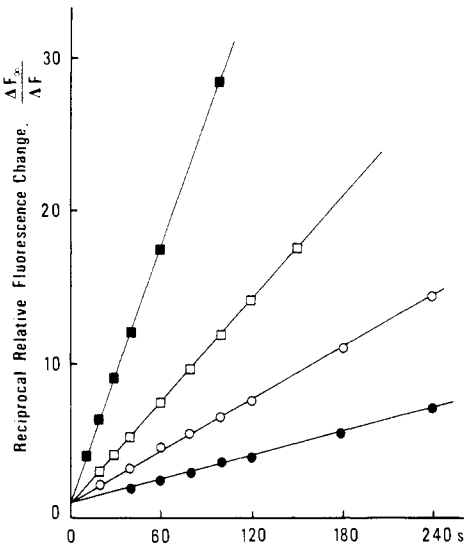


FIGURE 8: Kinetics of change in intrinsic fluorescence measured for the interaction of plasmin and $\alpha_2$-macroglobulin at various equal concentrations of the proteins. The ratio of the fluorescence change finally obtained, $\Delta F_\infty$, and that obtained at time $t$, $\Delta F$, is plotted against time. Protein concentrations were 50 (●), 100 (○), 200 (□), and 500 nM (■). The rate equation $A_0/A = ktA_0 + 1$, which corresponds to a reaction A + B $\xrightarrow{k}$ C when $A_0 = B_0$ and $C_0 = 0$, adequately describes these reactions. From the slopes of the lines a $k$ value $k_{1(P)} = 5 \times 10^5$ M$^{-1}$ s$^{-1}$ was obtained.

reaction step (Figure 7). This latter reaction apparently is a second-order reaction step with respect to the concentration of $\alpha_2$-macroglobulin (viz., 1:1 plasmin–$\alpha_2$-macroglobulin complex) and that of residual plasmin, with a rate constant $k_{2(P)} = 2 \times 10^3$ M$^{-1}$ s$^{-1}$.

The interaction of plasmin with $\alpha_2$-macroglobulin was further investigated by measuring the *final* changes in intrinsic fluorescence of $\alpha_2$-macroglobulin (0.100 $\mu$M) at various concentrations of plasmin and the *time course* of those changes at a number of equal concentrations of plasmin and $\alpha_2$-macroglobulin. Our results are similar to those of Straight & McKee (1982). The initial Δ(fluorescence)/Δ(plasmin) was close to 1.0. Very little change in fluorescence occurred beyond the 1:1 equivalence point (data not shown). Presumably formation of the 1:1 $\alpha_2$-macroglobulin–plasmin complex, of which no more than two thiol esters are cleaved, leads to almost complete conformational change of the entire $\alpha_2$-macroglobulin molecule as monitored by the change in intrinsic fluorescence. Cleavage of the second pair of thiol esters upon interaction with a second plasmin molecule apparently does not affect the conformation any further. Also, binding of the first trypsin molecule to $\alpha_2$-macroglobulin apparently is sufficient to effect the complete conformational change (Van Leuven et al., 1981; Sottrup-Jensen et al., 1983b; Gonias & Pizzo, 1983; Howell et al., 1983).

Plotting the reciprocal of the relative changes in intrinsic fluorescence obtained as a function of time at a number of equal concentrations of plasmin and $\alpha_2$-macroglobulin (Figure 8) shows that the time course of the reaction nicely fits that of a second-order reaction between plasmin and $\alpha_2$-macroglobulin with a rate constant $k_{1(P)} = 5 \times 10^5$ M$^{-1}$ s$^{-1}$. When trypsin interacts with $\alpha_2$-macroglobulin in similar experimental conditions, the changes in intrinsic fluorescence were unmeasurably fast. As judged from our results the generation of the first pair of thiol groups and the change in intrinsic fluorescence are events that occur apparently simultaneously. Table II shows the number of bait regions cleaved in plasmin–$\alpha_2$-macroglobulin complexes after 1- and 60-min incubations of plasmin and $\alpha_2$-macroglobulin (molar ratios 1.0, 2.0, and 3.0). The extent of cleavage found here is somewhat lower than that reported by Straight & McKee (1982), but apparently one bait region is cleaved in 1:1 plasmin–$\alpha_2$ macroglobulin complexes and two are cleaved in 2:1 complexes, and the first bait region cleavage reaction is relatively fast (possibly determined by $k_{1(P)}$) compared to the second (apparently determined by $k_{2(P)}$). The interaction of plasmin and $\alpha_2$-macroglobulin apparently follows a reaction pattern similar to that of trypsin and $\alpha_2$-macroglobulin at very low concentrations of free, active trypsin (<0.1 $\mu$M) but is different from that of trypsin and $\alpha_2$-macroglobulin at concentrations greater than that.

Table II: Extent of Bait Region Cleavage in $\alpha_2$-Macroglobulin-$^{125}$I-Labeled Plasmin Complexes[a]

| [$^{125}$I-labeled plasmin]/[$\alpha_2$M] (mol/mol) | incubation time (min) | % bait region cleavage[b] |
|---|---|---|
| 1.0 | 1 | 22 |
| 1.0 | 60 | 27 |
| 2.0 | 1 | 30 |
| 2.0 | 60 | 45 |
| 3.0 | 1 | 34 |
| 3.0 | 60 | 49 |

[a] Under various conditions, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. [b] 100% = four bait regions per mole.

*General Mechanism of $\alpha_2$-Macroglobulin–Proteinase Interactions.* The formation of a tight, essentially irreversible complex between $\alpha_2$-macroglobulin and a proteinase is a complex, multistep reaction, which involves subunit cleavage, gross conformational changes, and activation and ultimately cleavage of the internal thiol esters of $\alpha_2$-macroglobulin. $\alpha_2$-Macroglobulin is a new example of a protein regulated by specific limited proteolysis. Figure 9 shows a reaction scheme that illustrates the various alternative reactions that $\alpha_2$-macroglobulin may undergo when it forms complexes with proteinases.

The cleavage of *one* bait region seems to be prerequisite for the formation of tightly bound $\alpha_2$-macroglobulin–proteinase complexes. If this step is rate determining, a final 1:1 complex with three intact bait regions, but only one pair of thiol groups, is generated. Apparently this complex contains an entirely conformationally changed $\alpha_2$-macroglobulin molecule. If the cleavage of one bait region is *not* rate determining and the enzymic cleavage of bait regions is very fast, more than one bait region is cleaved before the proteinase is finally bound and further cleavages are thus prevented. Such $\alpha_2$-macroglobulin–proteinase complexes show four thiol groups per molecule, and both 1:1 and 1:2 complexes of this kind may be formed depending on the ratio of $\alpha_2$-macroglobulin to proteinase. These two possibilities are exemplified by the 1:1 complexes between $\alpha_2$-macroglobulin and plasmin on the one hand and $\alpha_2$-macroglobulin and trypsin (micromolar concentrations) on the other hand. When $\alpha_2$-macroglobulin–trypsin complexes are formed at very low concentrations of trypsin, the route of complex formation is similar to that shown by $\alpha_2$-macroglobulin and plasmin.

In enzyme kinetic terms the $k_1$ values reported here for trypsin- and plasmin-catalyzed cleavage of one bait region of native $\alpha_2$-macroglobulin should be equivalent to the $k_c/K_m$ values of those reactions. Evidently, the bait region is a good substrate of trypsin but only a moderately good substrate of plasmin. The lack of lysyl residues in the bait region (Sottrup-Jensen et al., 1983a) could in part explain this, since plasmin prefers lysyl for arginyl bonds (Christensen & Ipsen, 1979). The results presented suggest that the overall rates of $\alpha_2$-macroglobulin–proteinase interactions, which may differ

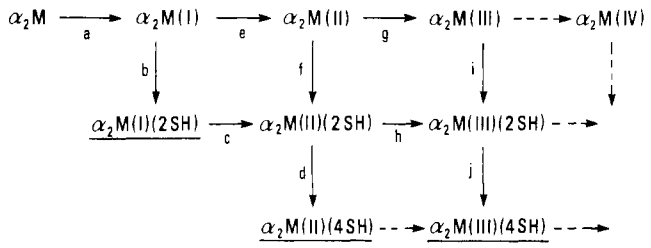


FIGURE 9: Reaction scheme illustrating the proposed alternative conversion routes that $\alpha_2$-macroglobulin may follow when it reacts with a proteinase to form $\alpha_2$-macroglobulin–proteinase complexes. Each horizontal reaction step is a proteolytic cleavage step in which one of the four $\alpha_2$-macroglobulin subunits is cleaved at the bait region. Each vertical reaction step is a rearrangement step leading to the generation of one pair of thiol groups with concomitant inhibition of an associated proteinase molecule, if such a molecule is present. The reacting proteinase molecules are not shown. The number of cleaved bait regions is indicated by (I), (II), etc. The number of generated thiol groups is indicated by (2SH) and (4SH). The various types of $\alpha_2$-macroglobulin modifications found in final $\alpha_2$-macroglobulin–proteinase complexes are underlined. In 1:1 plasmin–$\alpha_2$-macroglobulin complexes and in 1:1 trypsin–$\alpha_2$-macroglobulin complexes formed at very low concentrations of free, active trypsin the $\alpha_2$-macroglobulin apparently is of the type $\alpha_2$M(I)(2SH); that is, two thiol groups have been generated and one bait region has been cleaved. In 1:1 trypsin–$\alpha_2$-macroglobulin complexes formed at high concentrations of active trypsin the $\alpha_2$-macroglobulin apparently is mainly of the type $\alpha_2$M(II)(4SH), the same as in 2:1 plasmin–$\alpha_2$-macroglobulin complexes and in 2:1 trypsin–$\alpha_2$-macroglobulin complexes formed at a very low active trypsin concentration. Apparently some $\alpha_2$M-(III)(4SH) is also formed in these last cases. In 2:1 trypsin–$\alpha_2$-macroglobulin complexes formed at high concentrations of trypsin the type of $\alpha_2$-macroglobulin is mainly $\alpha_2$M(III)(4SH) or $\alpha_2$M-(IV)(4SH). It is assumed that the rates of the bait region cleavage steps are proportional to the concentration of free, active proteinase and that the rates of the rearrangement steps are not. At a branching point the further processing is determined by the ratio of the rates of the possible next reaction steps. For example, $\alpha_2$M(I) is primarily converted to $\alpha_2$M(II) if step e is faster than step b. The rate of step e increases when the concentration of active proteinase present is increased, whereas the rate of step b does not. A shift of reaction path should result at some point. The rate constants determined for trypsin were assigned as follows: for $k_1$, step a + step b, step a rate determining, therefore $k_{1(T)} = 2 \times 10^7$ $M^{-1}$ $s^{-1}$ of step a; for $k_2$, step c + step d, step c rate determining, therefore $k_{2(T)} = 1.3 \times 10^6$ $M^{-1}$ $s^{-1}$ of step c; for $k_{SH}$, step a + step e + step f + step d, step d and/or step f rate determining, therefore $k_{SH} = 0.2$ $s^{-1}$ of step d and/or f. The rate constants determined for plasmin were as follows: for $k_{1(P)}$, step a + step b, step a rate determining, therefore $k_{1(P)} = 5 \times 10^5$ $M^{-1}$ $s^{-1}$ of step a; for $k_{2(P)}$, step a + step b + step c + step d, step c rate determining, therefore $k_{2(P)} = 2 \times 10^3$ $M^{-1}$ $s^{-1}$ of step c.

by several orders of magnitude between individual proteinases, reflect the quality of the $\alpha_2$-macroglobulin bait regions as substrates for the interacting enzyme. This may explain that two different reaction pathways can be followed by enzymes when they form complexes with $\alpha_2$-macroglobulin.

For both trypsin and plasmin, 2:1 $\alpha_2$-macroglobulin complexes are formed even after a relatively slow generation of 1:1 complexes. The relatively low $k_2$ values determined for trypsin at very low concentrations of free, active trypsin and for plasmin probably reflect the change in conformation that takes place after the first bait region has been cleaved. Apparently this change affects the conformation or accesibility of the remaining bait regions. Under the conditions specified the cleavage of at least one additional bait region in the 1:1 proteinase–$\alpha_2$-macroglobulin complex in which two thiol groups have appeared results in the appearance of two new thiol groups, probably concomitantly with the formation of a binding site for the second proteinase molecule, so that 2:1 complexes form.

The reactions of $\alpha_2$-macroglobulin and proteinases appear from our results to follow two different reaction pathways dependent on the level of proteolytic activity. $\alpha_2$-Macroglobulin almost certainly plays a role in the regulation of extracellular proteolytic activity, and the two-pathway reaction mechanism seems to be well suited for that regulatory purpose: Low proteinase levels lead to 1:1 complexes, and the negative cooperativity results in a down-regulation of the effective resulting $\alpha_2$-macroglobulin activity 2 times that of the proteinase. High levels of proteinase lead to 2:1 complexes, thus lowering the concentration of the proteinase twice as much as that of $\alpha_2$-macroglobulin. This could be considered a kind of a buffering system.

It is striking that the rate of interaction between $\alpha_2$-macroglobulin and relatively specific proteinases like plasmin and thrombin is much lower than the rate of interaction between $\alpha_2$-macroglobulin and proteinases having a relatively broad specificity like trypsin, elastase, chymotrypsin, or cathepsin G as summarized by Travis & Salvesen (1983). This indicates that the main targets of $\alpha_2$-macroglobulin are relatively nonspecific proteinases, perhaps enzymes of cellular origin, which might appear in the blood or tissues as a result of cellular turnover. In addition, as pointed out by Starkey & Barrett (1977), $\alpha_2$-macroglobulin, by virtue of its unique bait region sequence (Sottrup-Jensen et al., 1981a; Mortensen et al., 1981), might constitute a defense barrier against those invasive pathogens and parasites that enter the body with the aid of secreted proteinases.

## Acknowledgments

Professor Ingemar Björk, Swedish University of Agricultural Sciences, Sweden, is thanked for the performance of the fluorescence measurements.

**Registry No.** STI, 9078-38-0; trypsin, 9002-07-7; plasmin, 9001-90-5; benzamidine, 618-39-3.

## References

Barrett, A. J. & Starkey, P. M. (1973) *Biochem. J. 133*, 709–724.

Chase, J., & Shaw, E. (1969) *Biochemistry 8*, 2212–2224.

Christensen, U. (1975) *Biochim. Biophys. Acta 397*, 459–467.

Christensen, U., & Ipsen, H.-H. (1979) *Biochim. Biophys. Acta 569*, 177–183.

Christensen, U., & Sottrup-Jensen, L. (1983) *Biochim. Biophys. Acta 747*, 263–275.

Debanne, M. T., Bell, R., & Dolovich, J. (1975) *Biochim. Biophys. Acta 411*, 295–304.

Deutsch, D. G., & Mertz, E. T. (1970) *Science (Washington, D.C.) 170*, 1095–1096.

Downing, M. R., Bloom, J. W., & Mann, K. G. (1978) *Biochemistry 17*, 2649–2653.

Dunn, J. T., & Spiro, R. G. (1967) *J. Biol. Chem. 242*, 5549–5555.

Gliemann, J., Larsen, T. R., & Sottrup-Jensen, L. (1983) *Biochim. Biophys. Acta 756*, 230–237.

Gonias, S. L., & Pizzo, S. V. (1983) *J. Biol. Chem. 258*, 14682–14685.

Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 457–464.

Harpel, P. C. (1973) *J. Exp. Med. 138*, 508–521.

Howard, J. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2235–2239.

Howell, J. B., Beck, T., Bates, B., & Hunter, M. J. (1983) *Arch. Biochem. Biophys. 222*, 261–270.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495.

Iwamoto, M., & Abiko, Y. (1970) *Biochim. Biophys. Acta 214*, 402–410.

Jones, J. M., Creeth, J. M., & Kekwick, R. A. (1972) *Biochem. J. 127*, 187–197.
Kaplan, J., & Nielsen, M. L. (1979) *J. Biol. Chem. 254*, 7329–7335.
Mortensen, S. B., Sottrup-Jensen, L., Hansen, H. F., Petersen, T. E., & Magnusson, S. (1981) *FEBS Lett. 135*, 295–300.
Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.
Sottrup-Jensen, L., Lønblad, P. B., Stepanik, T. M., Petersen, T. E., Magnusson, S., & Jörnvall, H. (1981a) *FEBS Lett. 127*, 167–173.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981b) *FEBS Lett. 128*, 127–132.
Sottrup-Jensen, L., Stepanik, T. M., Wierzbicki, D. M., Jones, C. M., Lønblad, P. B., Kristensen, T., Mortensen, S. B., Petersen, T. E., & Magnusson, S. (1983a) *Ann. N.Y. Acad. Sci. 421*, 41–60.
Sottrup-Jensen, L., Hansen, H. F., & Christensen, U. (1983b) *Ann. N.Y. Acad. Sci. 421*, 188–208.
Starkey, P. M., & Barrett, A. J. (1977) *Res. Monogr. Cell Tissue Physiol. 2*, 663–696.
Straight, D. L., & McKee, P. A. (1982) *Biochemistry 21*, 4550–4556.
Swenson, R. P., & Howard, J. B. (1979) *J. Biol. Chem. 254*, 4452–4456.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655–709.
Van Leuven, F., Cassiman, J.-J., & Van Den Berghe, H. (1979) *J. Biol. Chem. 254*, 5155–5160.
Van Leuven, F., Cassiman, J.-J., & Van Den Berghe, H. (1981) *J. Biol. Chem. 256*, 9016–9022.
Willingham, M. C., Maxfield, F. R., & Pasken, I. H. (1979) *J. Cell Biol. 82*, 614–625.
Wuepper, K. D., & Cochrane, C. G. (1972) *Proc. Soc. Exp. Biol. Med. 141*, 271–276.

# Tryptic Cleavage and Substructure of Bovine Cardiac Myosin Subfragment 1†

Dianne Applegate, Armando Azarcon, and Emil Reisler*

ABSTRACT: The method of limited tryptic proteolysis has been used to compare and contrast the substructure of bovine cardiac myosin subfragment 1 (S-1) to that of skeletal myosin S-1. While tryptic cleavage of cardiac S-1, like that of skeletal S-1, yields three fragments, the 25K, 50K, and 20K peptides, the digestion of cardiac S-1 proceeds at a 2-fold faster rate. The increased rate of cleavage is due entirely to an order of magnitude faster rate of cleavage at the 25K/50K junction of cardiac S-1 compared to that of skeletal, with approximately equal rates of cleavage at the 50K/20K junctions. Actin inhibits the tryptic attack at this latter junction, but its effect is an order of magnitude smaller for the cardiac than for the skeletal S-1. Furthermore, the tryptic susceptibility of the 50K/20K junction of cardiac S-1 in the acto–S-1 complex is increased in the presence of 2 mM MgADP. This effect is not due to partial dissociation of the cardiac acto–S-1 complex by MgADP. Our results indicate that in analogy to skeletal S-1, the cardiac myosin head is organized into three protease-resistant fragments connected by open linker peptides. However, the much faster rate of tryptic cleavage of the 25K/50K junction and also the greater accessibility of the 50K/20K junction in the cardiac acto–S-1 complex indicate substructural differences between cardiac and skeletal S-1.

Investigations of the properties of cardiac myosin often involve the application of techniques previously used to characterize the skeletal protein. The rationale of such studies on cardiac myosin is not only to further characterize this protein but also to compare and contrast its respective properties to those of skeletal myosin in an effort to find major determinants of the differing contractile properties of the two types of muscle.

For example, the enzymatic characterization of cardiac myosin has proceeded along such lines. Steady-state measurements indicate that the rate of MgATP hydrolysis by bovine cardiac myosin subfragment 1 (S-1)[1] at saturating concentrations of actin and ATP, $V_{max}$, is approximately 5-fold less than that of rabbit skeletal S-1 (Taylor & Weeds, 1976). Since the kinetic mechanism for the hydrolysis of ATP in the presence and absence of actin appears to be the same for both skeletal and cardiac subfragment 1 (S-1), the respective rate and equilibrium constants for the various steps in the kinetic scheme can be directly compared (Taylor & Weeds, 1975; Flamig & Cusanovich, 1983; Siemankowski & White, 1984). Such comparisons may have implications for the molecular mechanism of both skeletal and cardiac muscle contraction.

Likewise, the recent derivation from constructed clones of the amino acid sequence of portions of two rabbit cardiac myosin isozymes (Kavinsky et al., 1984) has allowed their direct comparison with the sequence obtained for rabbit skeletal myosin (Capony & Elzinga, 1981). Although the partial sequences of both clones exhibit extensive homology to the analogous regions of rabbit skeletal myosin, specific regions show a particularly high frequency of nonconservative substitutions. While these results are intriguing, the functional

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[1] Abbreviations: S-1, myosin subfragment 1; PMSF, phenylmethanesulfonyl fluoride; $Ap_5A$, $P^1,P^5$-di(adenosine-5′) pentaphosphate; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; LC-1, light chain 1.

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