Kinetics of the reaction of thrombin and α_2 -macroglobulin

Richard D. FEINMAN,* Anna I. YUAN, Steven R. WINDWER and Dalton WANG

Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, NY 11203, U.S.A.

The kinetics of the reaction of α_2 -macroglobulin (α_2 M) with human thrombin were studied by recording the appearance of thiol groups spectrophotometrically and by measuring the distribution of protein species by denaturing non-reducing gel electrophoresis. The goals were to study the relation between the formation of various covalent enzyme-inhibitor complex species and the appearance of free thiol, and from the kinetic analysis, to try to characterize the chemical nature of the protein complexes. The kinetics of thiol-group release were observed to be biphasic, the early phase showing second-order behaviour, results consistent with previous reports in the literature. The observed second-order rate constant for thiol-group release was found to be faster than the second-order rate constant for the disappearance of the band corresponding to native α_2 M on gel electrophoresis. This may be a reflection of the multiple products formed from the thioester. Alternatively, it is possible that covalent-bond formation is slower than some enzyme-induced change in the thioester centre, and this may be suggestive evidence for a reactive α_2 M centre that does not contain an intact thioester. The kinetics of covalent-bond formation were found to be consistent with the internal cross-link of several α_2 M chains by the bound proteinase, providing further evidence that the very-high- M_r species seen on gels may arise from dimers of the α_2 M molecule held together by covalent bonds to the enzyme.

INTRODUCTION

The function of formation of covalent complexes between α_2 M and proteolytic enzymes is not known, since non-covalent binding appears to be sufficient for the inactivation and clearance of the enzyme- α_2 M complex from the circulation (for reviews see: Barrett, 1981; Van Leuven, 1982*b*; Roberts, 1985). On the other hand, substantial covalent binding is found for most enzymes and is approx. 70–80% for trypsin (Salvesen & Barrett, 1980; Wu *et al.*, 1981; Salvesen *et al.*, 1981), and the site for covalent binding (an internal thioester between cysteine and glutamate residues) is required for the activity of α_2 M, at least for the human plasma protein. The potential for covalent-bond formation appears to be great, and evidence has been presented (Wang *et al.*, 1983, 1984) that bound enzymes are capable of forming more than one covalent bond to the subunits of α_2 M. This is the likely explanation for enzyme- α_2 M complexes of very slow mobility seen on dissociating gels, which, in some cases, account for the majority of the covalent complexes (Wang *et al.*, 1983; van der Graaf *et al.*, 1984). To examine further the role and nature of covalent complexes we performed kinetic experiments in which we compared the time course for formation of the various intermediates observed on non-reducing SDS/polyacrylamide-gel electrophoresis. We found that the kinetics were consistent with sequential formation of two covalent intermediates followed by parallel formation of two others. The last two intermediates are proposed to have unusual structure: dimers of whole α_2 M molecules held together by covalent bonds to the bound enzyme. We also measured the appearance of new thiol groups (due to destruction of the thioester) in parallel with the gel data. Comparison of the rate constant for thiol-group release

with that for loss of the native α_2 M band suggests that at least some of the thioesters are destroyed before covalent-bond formation. This is probably due to the (possibly rapid) hydrolysis of the thioester after binding of the enzyme. Alternatively, it may reflect the existence of a reactive centre for formation of covalent bonds that no longer contains the sulphur atom, e.g. an internal pyroglutamate. Such a reactive intermediate would be consistent with model studies of the reactive peptide (Khan & Erickson, 1982) and with the chemistry of this group in the native protein (Howard *et al.*, 1980).

EXPERIMENTAL

Preparation of α_2 M

The isolation and purification of α_2 M was carried out by a combination of zinc-chelate-agarose chromatography (Virca *et al.*, 1978) and chromatography on Cibacron Blue-Sepharose (Kurecki *et al.*, 1979). The details of the procedure are as follows.

Fresh human plasma (700 ml) containing 0.1 mg of soya-bean trypsin inhibitor/ml was diluted with 2 vol. of 0.1 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4. To this solution was added 50% (w/v) poly(ethylene glycol) to a final concentration of 4%. The mixture was stirred for 15 min on ice and centrifuged at 4200 *g* for 20 min. The poly(ethylene glycol) concentration of the supernatant was increased to 12% and the mixture was stirred for 15 min on ice and centrifuged at 4200 *g* for 20 min. The pellet was redissolved in 200 ml of 0.5 M-NaCl/20 mM-sodium phosphate buffer, pH 6.4, passed through a zinc-chelate-Sepharose column (2.5 cm $\times$ 60 cm) and washed with approx. 2 litres of 0.5 M-NaCl/20 mM-sodium phosphate buffer, pH 6.4. The α_2 M was

Abbreviations used: α_2 M, α_2 -macroglobulin; SDS, sodium dodecyl sulphate.

* To whom correspondence should be addressed.

eluted with 0.1 M-EDTA, pH 7.0. The pH of pooled active fractions was adjusted to 8.0 with NaOH. This material was passed through a Cibacron Blue–Sephacrose column (5 cm × 60 cm) pre-equilibrated with 50 mM-sodium phosphate buffer, pH 8.0. Fractions containing α_2 M were pooled and the pH was adjusted to 6.4 with 2 M- NaH_2PO_4 . Chromatography on a second zinc-chelate–Sephacrose column (2.5 cm × 19 cm) served to further purify and concentrate the material. The final material was eluted with 0.1 M-EDTA, pH 7.0, dialysed against 20 mM-sodium citrate buffer, pH 7.0, and stored in 2 ml portions at -70°C . The activity of α_2 M was assayed by protection of inhibition of esterase activity by soya-bean trypsin inhibitor, active-site titration and thiol-group release (Howard *et al.*, 1980; Wu *et al.*, 1981; Feinman *et al.*, 1983).

Proteins and reagents

Human α -thrombin was a gift from Dr. John W. Fenton, II, of the New York State Department of Health, Albany, NY, U.S.A. 4,4'-Dithiodipyridine was obtained from Sigma Chemical Co. and was used without further purification. Other chemicals were from commercial sources and were of reagent grade or better.

SDS/polyacrylamide-gel electrophoresis

Gels were prepared by the procedure of Davis (1964) as modified by Rosenberg *et al.* (1981). The separating and stacking gels were 3.5% and 3.0% acrylamide respectively, and were formed on a support of GelBond (FMC Corp.). Samples were precipitated with cold 10% (w/v) trichloroacetic acid, neutralized with NH_3 vapour and solubilized with 1% (w/v) SDS/25 mM-Tris/HCl buffer, pH 6.8, before being applied to gels. The stacking-gel buffer was 0.125 M-Tris/HCl, pH 6.8. Electrophoresis was carried out at 25 mA in Tris/glycine, pH 8.3, as the running buffer. Gels were stained with 0.1% Coomassie Brilliant Blue in 50% (v/v) methanol/10% (v/v) acetic acid, destained in 13% (v/v) acetic acid/20% (v/v) 2-methylpropan-2-ol and scanned in a Helena Quik Scan densitometer with automatic integrator.

Measurement of thiol groups

The appearance of thiol groups was measured with the reagent dithiodipyridine by monitoring the increase in absorbance at 324 nm (Grassetti & Murray, 1967). In initial studies of the appearance of thiol groups, we observed substantial precipitation of protein with the use of 5,5'-dithiobis-(2-nitrobenzoic acid) as thiol-group indicator.

Kinetic analysis

Second-order rate constants were determined on an Apple II+ microcomputer by using a non-linear-regression analysis as described by Wong *et al.* (1983). For thiol-group release, the rate constant for approach to the final slope is calculated.

RESULTS

Measurement of the appearance of thiol groups

Fig. 1 shows the kinetics of reaction of α_2 M with several different concentrations of human α -thrombin. The appearance of free thiol groups during the reaction

Fig. 1. Kinetics of appearance of thiol groups in the reaction of α_2 M with thrombin

The concentration of α_2 M was $1.38 \mu\text{M}$, and that of dithiodipyridine was 0.5 mM. For full experimental details see the text. The thrombin/ α_2 M ratio is indicated on each curve. Reactions were carried out at 25°C in Tris/HCl buffer, pH 8.0.

Fig. 2. Computed fit of kinetic data to second-order mechanism

+, Thiol-group release; ■, density of band 1.

was recorded spectrophotometrically with dithiodipyridine as an indicator. The reactions showed biphasic kinetics with a very slow final phase. Kinetic analysis was carried out by subtracting the extrapolated slow phase from the time-progress curves. The resulting data were found to follow second-order kinetics; a fitted curve is shown in Fig. 2. Apparent second-order rate constants for the initial phase at pH 8.0 are listed in Table 1, and it can be seen that these apparent constants depend on the initial thrombin concentration. This may be a reflection of different dependences on enzyme concentration of separate reactions of thioester centres (hydrolysis and formation of covalent bonds with enzymes). To try to correlate the appearance of these thiol groups with events at the thioester site, we carried out experiments to observe

Table 1. Second-order rate constants for α_2 M reaction with thrombin

Rate constants for the disappearance of band 1 on SDS/polyacrylamide-gel electrophoresis (k_1) and for the appearance of thiol groups (k_{SH}) were determined as described in the Experimental section and demonstrated in Fig. 2.

Thrombin/ α_2 M ratio	$10^3 \times k_1$ ($M^{-1} \cdot s^{-1}$)	$10^3 \times k_{SH}$ ($M^{-1} \cdot s^{-1}$)
0.5:1	0.87	5.52
1:1	2.70	5.52
	1.48	5.88
2:1	1.37	3.50
3:1	1.10	2.58
4:1	1.34	2.39

Fig. 3. SDS/polyacrylamide-gel electrophoresis of thrombin- α_2 M reactions

The enzyme/ α_2 M ratio was (a) 2:1 or (b) 4:1. For full experimental details see the text. Protein was stained with Coomassie Brilliant Blue.

directly the covalent complexes formed. The results of our studies of these protein species as revealed by gel electrophoresis are described below.

SDS/polyacrylamide-gel electrophoresis of thrombin- α_2 M complexes

To simplify the interpretation of the kinetic data, the behaviour of the thrombin- α_2 M system on SDS/polyacrylamide-gel electrophoresis (Wang *et al.*, 1983, 1984) is briefly summarized. There are five major bands. Band 1 arises from native inhibitor or non-covalent complexes and has an apparent mass of the half-molecule, M_r 375000. (Two pairs of subunits and the proteolysed

fragments of the subunits are disulphide-bonded and do not dissociate in SDS.) Band 2 is usually attributed to the half-molecule with covalently bound enzyme. Band 3 is assumed to be a covalently cross-linked α_2 M species of the size of the entire α_2 M molecule (M_r greater than 750000) although it has anomalously high mobility (Wang *et al.*, 1984). Bands 4 and 5 have not been characterized but are believed to be separated by similar large differences in mass. Autoradiography of reactions with labelled proteinases show that all but band 1 are enzyme-containing species. The covalent complexes in these experiments constitute about 60% of the total binding of enzyme (Wang *et al.*, 1981, and unpublished work).

Fig. 4. Kinetics of proteins observed on SDS/polyacrylamide-gel electrophoresis

The Figure shows the integrated density from densitometry of electrophoretic bands in Fig. 3(b) (thrombin/ α_2 M ratio 4:1). Band 4 kinetics are essentially parallel to band 5 kinetics, but are not shown, for clarity (see Fig. 6).

Kinetics of formation of enzyme- α_2 M complexes observed by polyacrylamide-gel electrophoresis

Fig. 3 shows results of typical experiments in which thrombin and α_2 M were allowed to react under the same conditions as in Fig. 1. At various times, samples were removed, immediately denatured with trichloroacetic acid and subjected to electrophoresis on polyacrylamide. The integrated densities of the bands are plotted in Fig. 4 for a typical experiment. The kinetics of appearance of free thiol groups under these conditions are shown (broken line) on the same plot for comparison. The important features of these kinetic runs are as follows.

(1) The disappearance of band 1 follows second-order kinetics; a computer fit of typical data is shown in Fig. 2. Rate constants, k_1 , for different experiments are tabulated in Table 1, where they can be compared with those for thiol-group release. The disappearance of band 1 is second-order for the time of the experiment and does not show the slow second phase observed for thiol-group release. The two kinetic steps differ, as well, in that k_1 is not dependent on the enzyme, whereas k_{SH} , as described above, must contain terms in the concentration of reactants. The apparent constant k_1 is also invariably lower than the value for thiol-group release under the same conditions.

(2) Band 2 reaches a maximum with time and then decays to a final value. The effect of the initial conditions on the behaviour of this species is shown in Fig. 5. As the thrombin/ α_2 M ratio increases, the time to appearance of the peak is shortened and the height of the peak increases. This behaviour is consistent with a series reaction in which the first step is concentration-dependent and a second product arises from the intermediate. It is therefore reasonable to assume that the intermediate containing band 2 arises from the species containing band 1, and then goes on to form a new product.

(3) The kinetic behaviour of band 3 is less predictable than that of the other bands; for some concentrations of reactants it reaches a plateau, whereas in other cases there is a clear maximum followed by a decay. From the

Fig. 5. Time-dependence of band 2 from SDS/polyacrylamide-gel electrophoresis of reactions of α_2 M with thrombin

The Figure shows data from experiments of the type shown in Figs. 3 and 4. The thrombin/ α_2 M ratio is shown for each curve. Values are normalized for the total initial amount of band 1 for each run.

Fig. 6. Time-dependence of bands 4 and 5 from SDS/polyacrylamide-gel electrophoresis

The thrombin/ α_2 M ratio is indicated in the upper left-hand corner of each part of the Figure. Values are normalized for the total initial amount of band 1 for each run.

kinetics of band 2 described above and the chemical evidence, it is most reasonable to assume that the species giving rise to band 3 arises from band 2. An unusual feature of the kinetics, however, is that the rate of appearance of band 3 is initially very rapid. This means that, if species 3 does, in fact, arise from species 2, then formation of species 2 must be rate-determining for the overall process, since band 3 appears at almost the same rate. This may partially explain the stoichiometry of 2:1 or greater usually observed in the literature for thiol-group release compared with proteolysis (Sottrup-Jensen *et al.*, 1980; Straight & McKee, 1984), i.e. at least two processes involving the release of thiol groups are going on during

covalent-bond formation. (Presumably hydrolysis also contributes to this phenomenon.) If this is correct, then, for those reactions in which band 3 reaches a plateau, there is a steady state of competing reactions: continued formation of band 3 from band 2 and its disappearance to form other species. The kinetic behaviour of bands 4 and 5, described below, suggests that these are, in fact, the products of the reaction of species 3.

(4) The kinetics of bands 4 and 5 show a distinct lag phase, characteristic of the appearance of product in a series reaction (Fig. 6). An important feature of these reactions is that the kinetics of appearance of bands 4 and 5 are parallel for most of the reaction and, within experimental error, there is approximately the same inflexion point. We conclude that these forms arise by the same formal mechanism and that the two series reactions have similar rate constants.

DISCUSSION

Multivalent enzyme- α_2 M complexes

A model that incorporates the major features of the kinetics of thiol-group appearance and formation of covalent complexes in the thrombin- α_2 M reaction is shown in Scheme 1. It provides for the formation of univalent complexes, which react further to form multivalent species. These latter structures, in which more than one subunit or molecule of protein are cross-linked via covalent bonds to a second protein, are, to our knowledge, unique in biochemistry. The idea that bound proteinases might form a 'protein bridge' between subunits of α_2 M was first proposed by Krebs *et al.* (1978) on the basis of the ability of trypsin to decrease the dissociation of α_2 M into subunits as observed in the

ultracentrifuge. The major evidence for structures in which two proteolysed subunits were cross-linked by two bonds to an enzyme molecule comes from studies of two-dimensional electrophoresis (Wang *et al.*, 1983). This idea is also supported by experiments performed by Gonias & Pizzo (1983*a,b*), which showed high- M_r enzyme-containing species in the reaction of trypsin with native α_2 M but not with dissociated half-molecules. The fact that the formation of these complexes is decreased in reactions with enzymes in which the lysine-residue amino groups have been blocked (Wang *et al.*, 1984) suggests that the linkages are an amide of the type involved in univalent complexes (Sottrup-Jensen *et al.*, 1983).

Kinetic model

The initial step in the reaction of thrombin with α_2 M is assumed to be the formation of a non-covalent complex. This step is presumably very fast, and the product is not distinguishable from the native inhibitor on SDS/polyacrylamide-gel electrophoresis (migrates with the native half-molecule in band 1). Non-covalent complexes and the native inhibitor are represented in Scheme 1 by a structure shown in brackets. It is widely held that during α_2 M reactions the enzymes can be physically 'trapped' in a clathrate-type structure in which the α_2 M surrounds the enzyme (Barrett & Starkey, 1973; Barrett *et al.*, 1979). If no covalent bond were formed such species would appear in band 1, and would be included in the composite structure shown in brackets. It has been argued that covalent complexes may also have a 'trapped' structure, but in the absence of any molecular indicator for this state the model does not address this directly.

Scheme 1. Model for the reaction of α_2 M with thrombin

Ellipses represent subunits of α_2 M; half-ellipses represent proteolysed subunits; filled circles represent thrombin; lines between α_2 M ellipses or half-ellipses represent disulphide bonds; lines between enzyme and α_2 M species represent covalent amide bonds (between lysine and glutamate residues). Numbered species correspond to the numbers of the bands on SDS/polyacrylamide-gel electrophoresis (Fig. 3), and broken arrows indicate the dissociation in SDS. Species 3 and 5 do not dissociate in SDS. The first structure in brackets represents a composite of native α_2 M and (possibly proteolysed) non-covalent enzyme complexes; the actual stoichiometry of hydrolysis of α_2 M with enzyme binding is not shown. For further discussion see the text.

The model in Scheme 1 proposes that non-covalent complexes give rise to the univalent species with apparent rate constant k_1 (see the Experimental section). This species now rapidly undergoes a transformation into species 3. In particular, structure 3 arises by intramolecular attack (within species 2) of α_2 M-bound enzyme lysine-residue amino groups on thioesters of another subunit of the α_2 M molecule, as previously proposed (Wang *et al.*, 1983, 1984). The major novel feature of the model is that it proposes that form 3 can now undergo an intermolecular reaction with another α_2 M molecule, to form the dimeric species that give rise to bands 4 and 5 on electrophoresis. Whether the product will be type 4 or type 5 will be determined by whether the reacting second molecule is of the type species 2 or species 3. In other words, species 2 and 3 are kinetically identical for reaction with another molecule of species 3, the rate presumably being controlled by the collision frequency; the product distribution will be determined by the relative amounts of species 2 and 3. Evidence supporting this idea is the observation (Fig. 6) that the rates of appearance of species 4 and 5 are essentially parallel, with approximately the same inflexion point. The dependence of the band 5/band 4 ratio on initial conditions, shown in Fig. 6, can be rationalized in terms of the higher thrombin/inhibitor stoichiometry in complex 5 making it favoured at high enzyme concentration. Finally, to be consistent with previously published results (Wang *et al.*, 1983), which showed that band 5 gave rise to complexes of M_r significantly greater than 260000 after reduction, the model includes tervalent species.

The temporal and causal links between the separate aspects of the reaction of α_2 M with proteinases are not known. Generally, conformational changes and the release of thiol groups have been found to be concomitant. Also, Christensen & Sottrup-Jensen (1984) have shown parallel release of thiol groups, covalent binding and cleavage of the 'bait' region of the inhibitor, and the concentration of native α_2 M remaining after methylamine treatment follows the same kinetics as residual enzyme binding (Van Leuven *et al.*, 1982a). Likewise, Straight & McKee (1982) found that the second-order rate constant for the conformational change measured by intrinsic fluorescence was the same as the constant for total enzyme binding. This constant $2.5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ measured at pH 7.4 is in good agreement with our values of 2.39×10^3 – $5.88 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ determined at pH 8.0 for the appearance of thiol groups. It is significant that Fig. 2 and Table 1 show that thiol-group release and the disappearance of band 1 (from native α_2 M and non-covalent complexes) are different. The most likely explanation is that several different reactions of the thioester occur simultaneously, only one of which correlates with the formation of the first covalent intermediate. Thus, if (non-productive) hydrolysis of the thioester were significantly faster than formation of covalent bonds, this might account for an apparent high rate constant. Alternatively, this observation is suggestive of a mechanism whereby the initial reaction with proteinase causes conversion of the thioester into a reactive intermediate in which the thiol group has already been lost. This intermediate would then, in turn, react with nucleophiles. In this regard, Sottrup-Jensen *et al.* (1980, 1983) showed that, in enzyme-treated α_2 M samples, residual covalent-binding ability decreased less after 15 min than predicted by the appearance of free thiol

groups. An active glutamate-residue centre derived from the thioester, an endopyroglutamate (imide) group formed from the nitrogen atom of the neighbouring peptide bond, was identified by model studies of the reactive hexapeptide (Khan & Erickson, 1982; Erickson & Khan, 1983). Evidence for the involvement of the endopyroglutamate form in heat fragmentation of α_2 M and its relation to the thioester in the native protein has been presented by Howard and co-workers (Howard *et al.*, 1980; Howard, 1983).

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