Rudolph, R. (1990) in *Modern Methods in Protein and Nucleic Acid Analysis* (Tschesche, H., Ed.) pp 149–171, Walter de Gruyter, Berlin.
Rudolph, R., & Jaenicke, R. (1976) *Eur. J. Biochem. 63*, 409–417.
Saxena, V. P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015–5023.
Semisotnov, G. V., Rodionova, N. A., Kutyshenko, V. P., Ebert, B., Blanck, J., & Ptitsyn, O. B. (1987) *FEBS Lett. 224*, 9–13.
Teipel, J. W., & Koshland, D. A., Jr. (1971) *Biochemistry 10*, 792–805.
Wetlaufer, D. B., Johnson, E. R., & Clauss, L. M. (1974) in *Lysozyme* (Osserman, E. F., Canfield, R. E., & Beychok, S., Eds.) pp 269–280, Academic Press, New York and London.
Yutani, K., Yutani, A., Imanishi, A., & Isemura, T. (1968) *J. Biochem. (Tokyo) 64*, 449–453.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1979) *Biochemistry 18*, 5567–5571.

# Reaction of Proteinases with $\alpha_2$-Macroglobulin: Rapid-Kinetic Evidence for a Conformational Rearrangement of the Initial $\alpha_2$-Macroglobulin–Trypsin Complex†

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ABSTRACT: The kinetics of the reaction of trypsin with $\alpha_2$M were examined under pseudo-first-order conditions with excess inhibitor. Initial studies indicated that the fluorescent dye TNS is a suitable probe for monitoring the reaction over a wide concentration range of reactants. Titration experiments showed that the conformational changes associated with the binding of trypsin to $\alpha_2$M result in an increased affinity of the inhibitor for TNS. Two distinct phases were observed when this dye was used to monitor the progress of the reaction. Approximately half of the fluorescence signal was generated during a rapid phase, with the remainder generated during a second, slower phase. The observed pseudo-first-order rate constant of the first phase varied linearly with the concentration of $\alpha_2$M up to the highest concentration of inhibitor used, whereas the rate constant of the second phase was independent of $\alpha_2$M concentration. The data fit a mechanism in which the association of trypsin with $\alpha_2$M occurs in two consecutive, essentially irreversible steps, both leading to alterations in TNS fluorescence. The initial association occurs with a second-order rate constant of $(1.0 \pm 0.1) \times 10^7$ $M^{-1}$ $s^{-1}$ and is followed by a slower, intramolecular conformational rearrangement of the initial complex with a rate constant of $1.4 \pm 0.2$ $s^{-1}$. The data are consistent with a previously proposed model for the reaction of proteinases with $\alpha_2$M [Larsson et al. (1989) *Biochemistry 28*, 7636–7643]. In this model, once an initial 1:1 $\alpha_2$M–proteinase complex forms, the complex either can react with a second proteinase molecule or can undergo a conformational rearrangement that generates a complex greatly reduced in its ability to bind additional proteinase. The detection of two kinetic phases in the present study and the excellent agreement between the range of 1–2 $s^{-1}$ predicted from modeling experiments for the magnitude of this conformational change and the value of 1.4 $s^{-1}$ obtained for the slow phase provide evidence supporting this model.

$\alpha_2$-Macroglobulin ($\alpha_2$M)[1] is a plasma glycoprotein that is capable of reacting with numerous proteinases (Barrett, 1981). This molecule is one member of a family of proteins that contain thiol ester bonds (Sottrup-Jensen et al., 1985). Reaction of $\alpha_2$M with proteinases is initiated by a selective cleavage (Harpel, 1973) at a specific "bait" region of the $\alpha_2$M subunit, which activates the molecule by inducing a series of conformational changes within $\alpha_2$M that can be detected by a variety of physical techniques (Björk & Fish, 1982; Gonias et al., 1982; Strickland & Bhattacharya, 1984; Strickland et al., 1984). These conformational alterations result in activation of the thiol ester bonds which increases the reactivity of these bonds toward nucleophilic molecules (Sottrup-Jensen et al., 1981), in a reduction of the activity of the proteinase toward large macromolecular substrates (Barrett & Starkey, 1973), and in the generation of regions on $\alpha_2$M (Imber & Pizzo, 1981; Kaplan & Nielsen, 1979) that interact with a specific cell surface receptor. This receptor has been recently identified

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[1] Abbreviations: $\alpha_2$M, $\alpha_2$-macroglobulin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid.

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as a large cell surface glycoprotein (Moestrup & Gliemann, 1989; Ashcom et al., 1990; Strickland et al., 1990).

$\alpha_2$M contains four identical subunits whose primary structure is known (Sottrup-Jensen et al., 1984). To date, the arrangement of proteinase binding sites within the molecule has not been elucidated. However, reports that $\alpha_2$M "half-molecules" that appear capable of binding proteinase can be isolated either by limited reduction (Gonias & Pizzo, 1983) or by urea treatment (Liu et al., 1987) have led to the conclusion that $\alpha_2$M can bind maximally 2 mol of proteinase per inhibitor molecule. Binding studies have demonstrated that this maximal capacity is used by $\alpha_2$M in binding certain proteinases, such as trypsin and chymotrypsin (Ganrot, 1966; Swenson & Howard, 1979; Björk et al., 1984), but is not used with other proteinases, such as thrombin, where only 1 mol of enzyme is bound (Steiner et al., 1985). Binding experiments with brinase (Larsson et al., 1988) and collagenase (Sottrup-Jensen & Birkedal-Hansen, 1989) have demonstrated that there is no clear connection between the size of the proteinase and the final binding stoichiometry.

While occupation of the two $\alpha_2$M–proteinase binding sites does not appear to be strictly correlated with the size of the proteinase, the binding stoichiometry does depend upon the conditions of the reaction (Salvesen et al., 1983; Christensen & Sottrup-Jensen, 1984; Larsson et al., 1989). Larsson et al. (1989) noted that the amount of trypsin bound to $\alpha_2$M was dependent on the concentration of the reactants. As these concentrations were decreased from the micromolar to nanomolar level, the maximal binding ratio thus decreased from 2:1 to 1.3:1. Studies in which 1:1 $\alpha_2$M–proteinase complexes were characterized have revealed additional insight into the binding mechanism (Strickland et al., 1988). These complexes had two of the four $\alpha_2$M subunits cleaved, had changes in TNS fluorescence that were approximately half of those measured for complexes containing 2 mol of proteinase, and had an intermediate mobility between native $\alpha_2$M and 1:2 $\alpha_2$M–proteinase complexes in polyacrylamide gel electrophoresis under nondenaturing conditions. The alterations thus appeared restricted to one of the two functional units of the inhibitor. Despite this, these complexes were found to be limited in their ability to bind additional proteinases.

A simple model was described to account for these observations (Larsson et al., 1989). This model predicts that once a 1:1 $\alpha_2$M–proteinase complex forms, the complex can either react with additional proteinase or undergo a conformational rearrangement to yield a complex with greatly reduced proteinase binding ability. Modeling experiments estimated the magnitude of the rate constant of this conformational change to be 1–2 $s^{-1}$. The purpose of the present investigation was to examine the reaction of trypsin with $\alpha_2$M by stopped-flow techniques to verify the validity of the proposed mechanism.

## Materials and Methods

*Proteins.* $\alpha_2$M was isolated from fresh frozen human plasma (Washington Regional Blood Services, American Red Cross) as previously described (Strickland et al., 1988). Immunoaffinity chromatography (Strickland et al., 1988) was employed when necessary to remove trace amounts of inactive $\alpha_2$M. Bovine trypsin (type III, twice crystallized) was obtained from Sigma Chemical Co. (St. Louis, MO). $\beta$-Trypsin was isolated by affinity chromatography of the commercial preparation on agarose-linked soybean trypsin inhibitor (Robinson et al., 1971; Yung & Trowbridge, 1975). Active-site titration with *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (Chase & Shaw, 1970) gave 0.89 mol of active site per mole of enzyme. Protein concentrations were obtained by absorption measurements at 280 nm. The values for $A_{1\%}^{280nm}$ and molecular weights used were as follows: $\alpha_2$M, 8.93 and 718 000 (Jones et al., 1972; Hall & Roberts, 1978); trypsin, 15.4 and 23 000 (Robinson et al., 1971; Walsh & Neurath, 1964).

*TNS Titrations.* TNS (Molecular Probes, Eugene, OR) was dissolved in *N*,*N*-dimethylformamide. The concentration of the stock solution was determined to be 5.0 mM by absorption measurements using a molar extinction coefficient of $1.89 \times 10^4$ at 317 nm (McClure & Edelman, 1966). To minimize the inner filter effect, the dye was excited at 385 nm, while emission was monitored at 420 nm. Seventy microliters of the dye was added continuously at a rate of 10 $\mu$L/min via a computer-controller motor-driven syringe to a stirred cuvette at 25 °C containing 2.0 mL of $\alpha_2$M in 50 mM Hepes, 0.1 M NaCl, and 2 mM EDTA, pH 7.4. Data were collected at 5-s intervals using an SLM 8000-C fluorometer. A blank sample, containing buffer, was also titrated with TNS, and the small change in fluorescence was subtracted from all other samples. The values were then corrected for the inner filter effect of the added ligand by using the following relationship (McClure & Edelman, 1967): $F_{corr} = F_{obs}[2.303\epsilon_{385}L_0/(1 - 10^{-\epsilon_{385}L_0})]$, where $L_0$ is the total TNS concentration, $\epsilon_{385}$ is the molar absorption coefficient at the excitation wavelength, and $F_{obs}$ and $F_{corr}$ refer to the observed and corrected fluorescent intensities, respectively. The data were analyzed by nonlinear regression using the relationship:

$$\Delta F = \Delta F_{max}\left(\frac{[L]_T}{K_d + [L]_T}\right) \quad (1)$$

where $\Delta F$ is the corrected change in fluorescence, $\Delta F_{max}$ is the maximum fluorescence change at saturation of $\alpha_2$M with TNS, $[L]_T$ is the molar concentration of total TNS, and $K_d$ is the dissociation constant. $\Delta F_{max}$ and $K_d$ were treated as variable parameters in the fitting procedure. Application of this analysis to the data assumes that the concentration of bound TNS is small relative to the total concentration of TNS. This was approximately true in all titrations, especially at higher ligand concentrations, where the concentration of free TNS approaches the total TNS concentration.

*Kinetic Measurements.* The kinetics of association between $\alpha_2$M and trypsin were studied under pseudo-first-order conditions, i.e., at a 1:10 molar ratio of trypsin to $\alpha_2$M. The reactions were performed at 25.0 °C in a buffer of 50 mM Hepes, 0.1 M NaCl, and 2 mM EDTA, pH 7.4, containing 50 $\mu$M TNS. Reaction progress was monitored by fluorescence measurements in a Hi-Tech SF-4 stopped-flow spectrophotometer (Hi-Tech, Salisbury, Wilts, England), with a dead time of approximately 2 ms. Excitation was at 315 nm with a 150-W xenon lamp, and emission was observed through a cutoff filter with 50% transmission at 400 nm. Data were collected as previously described (Björk et al., 1989).

*Data Analysis.* The data were analyzed by iteratively fitting to curves described by the appropriate equations. Fitting was accomplished on a VAX 8350 by nonlinear regression analysis using the software program RS1 (BBN Research Systems, Cambridge, MA). Initial estimates of the parameters were usually derived from a grid search, and the final values were derived from the Marquardt–Levenberg method until a least-squares solution was reached.

## Results

*Binding of TNS to $\alpha_2$M, Trypsin, and $\alpha_2$M–Trypsin Complexes.* Kinetic measurements in this study were carried out under pseudo-first-order conditions with excess $\alpha_2$M over trypsin. To monitor the reaction under these conditions, a

Table I: Binding Parameters from Titrations of $\alpha_2$M–Trypsin Complexes and $\alpha_2$M with TNS[a]

| protein | concn ($\mu$M) | $K_d$ ($\mu$M) | $\Delta F_{max}$ |
|---|---|---|---|
| $\alpha_2$M–trypsin | 1 | 102 ± 2 | 11.9 ± 0.1 |
| | 2 | 92 ± 3 | 19.3 ± 0.3 |
| | 4 | 130 ± 4 | 43.3 ± 0.7 |
| $\alpha_2$M | 1 | 227 ± 12 | 8.1 ± 0.3 |
| | 2 | 194 ± 3 | 17.3 ± 0.2 |
| | 4 | 229 ± 4 | 34.3 ± 0.4 |

[a] Values are given with their standard deviations determined by nonlinear regression analysis.

sensitive probe is required that is capable of detecting small amounts of $\alpha_2$M–proteinase complex in the presence of relatively large amounts of the intact molecule. An increase in intrinsic fluorescence accompanies the conversion of native $\alpha_2$M to the proteolyzed molecule (Björk & Fish, 1982), and experiments monitoring tryptophan fluorescence therefore were attempted. However, these experiments did not give interpretable data over a sufficiently wide concentration range, due to the high fluorescence background from excess $\alpha_2$M present during the reaction. Previous studies have demonstrated that the fluorescent dye TNS is sensitive to conformational changes in $\alpha_2$M (Strickland & Bhattacharya, 1984; Steiner et al., 1987; Björk et al., 1985), the formation of $\alpha_2$M–proteinase complexes being accompanied by a substantial enhancement of the fluorescence of the bound dye. Since preliminary experiments indicated that reproducible and interpretable kinetic data were obtained by monitoring TNS fluorescence changes, initial studies were performed to characterize the binding of this probe to $\alpha_2$M, $\alpha_2$M–trypsin complexes, and trypsin alone.

Different concentrations of $\alpha_2$M–trypsin complexes (Figure 1A) and intact $\alpha_2$M (Figure 1B) were titrated with TNS. Excellent fits of the data to a single class of binding sites were obtained for the binding of TNS to both the native and the proteolyzed molecule (Figure 1), giving dissociation constants that were independent of protein concentration (Table I). A $K_d$ of 108 ± 16 $\mu$M was determined for the binding of TNS to the $\alpha_2$M–trypsin complex, while the binding of TNS to the native molecule was considerably weaker, with a $K_d$ of 216 ± 16 $\mu$M. These values are different from those of 1.4 and 7.1 $\mu$M reported by Favaudon et al. (1987) for the binding of TNS to the $\alpha_2$M–chymotrypsin complex and to native $\alpha_2$M, respectively. In the latter study (Favaudon et al., 1987), the $K_d$ values were determined by extrapolation of data collected between 0.4 and 4.0 $\mu$M TNS, which is well below the dye concentrations required for saturation. This could have led to a large error in the $K_d$ values and may account for the differences between the two studies.

The fitting process also determines the maximal fluorescence enhancement ($\Delta F_{max}$) achieved at infinite concentration of TNS (Table I) and showed that the $\Delta F_{max}$ values were slightly higher for $\alpha_2$M–trypsin complexes than for $\alpha_2$M alone. The fluorescence titrations thus suggest that the conformational changes of $\alpha_2$M associated with binding of trypsin primarily result in an increased affinity of the inhibitor for TNS, although the mode of binding also appears to be somewhat altered. This interpretation is consistent with the spectral changes (i.e., fluorescence enhancement and blue shift) observed during this process (Strickland & Bhattacharya, 1984). Similar titration experiments showed that the binding of TNS to trypsin is very weak, with a $K_d > 500$ $\mu$M, and with a maximal fluorescence enhancement appreciably smaller than that given by either $\alpha_2$M or the $\alpha_2$M–trypsin complex. Overall, these experiments indicate that at a dye concentration around 50 $\mu$M, it should be possible to detect the generation of small

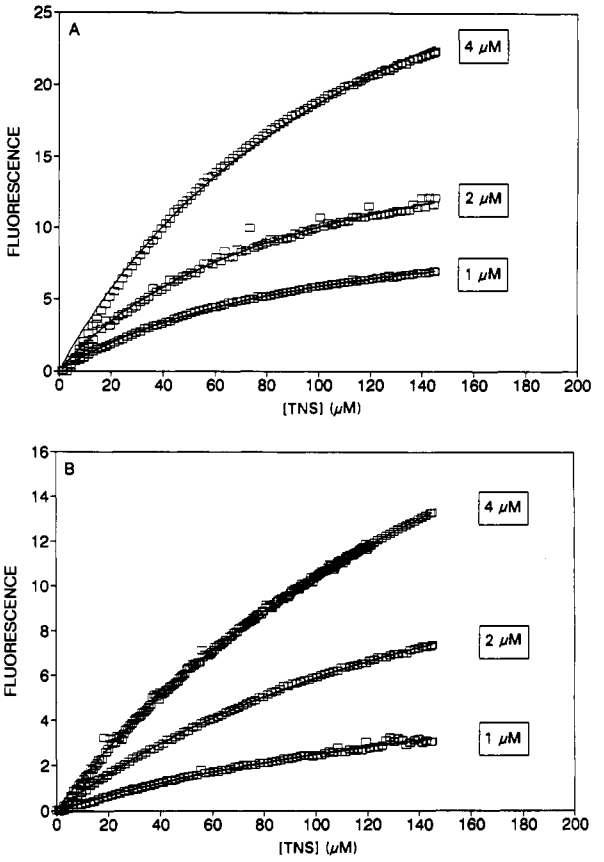


FIGURE 1: Titration of $\alpha_2$M–trypsin complex (A) and intact $\alpha_2$M (B) with TNS. The indicated amounts of protein were added to a stirred cuvette, and increasing amounts of TNS were then continuously added by a computer-controlled pump. Excitation was at 385 nm, while emission was monitored at 420 nm. The data were corrected for dilution and inner filter effects. The curves represent the best fits using nonlinear regression to a single class of binding sites.

amounts of $\alpha_2$M–trypsin complex in the presence of relatively large amounts of the native molecule, with TNS binding to trypsin negligibly affecting the results. This concentration of dye was therefore chosen for the kinetic studies.

*Kinetics of Association with $\alpha_2$M.* The use of TNS as a probe for monitoring conformational changes in $\alpha_2$M during the reaction with proteinases requires that dye binding be more rapid than these conformational changes. To substantiate this, stopped-flow experiments were conducted in which native $\alpha_2$M or $\alpha_2$M–trypsin complexes (1 $\mu$M) were mixed with TNS (50 $\mu$M). These experiments revealed that all of the changes in the fluorescence signal occurred within the mixing time of the instrument (~2 ms), showing that TNS binding to $\alpha_2$M conformers is considerably more rapid than the reactions being monitored.

The kinetics of the reaction between $\alpha_2$M and trypsin were studied in the presence of 50 $\mu$M TNS at a mole ratio of trypsin to $\alpha_2$M of 1:10 and with $\alpha_2$M concentrations varying between 1 and 11 $\mu$M. Representative data are shown in Figure 2. It is apparent from these results that the association of trypsin with $\alpha_2$M occurs in two distinct phases, a rapid phase (inset, Figure 2) being followed by a much slower reaction. It is also apparent from examination of the first-order plots (Figure 3) that approximately half of the signal change occurs during the first, rapid phase with the remainder being associated with the slower phase. Further examination of Figures 2 and 3 reveals that the rate of the rapid phase is dependent on the concentration of $\alpha_2$M, while the rate of the slower phase appears to be similar for all three concentrations of $\alpha_2$M.

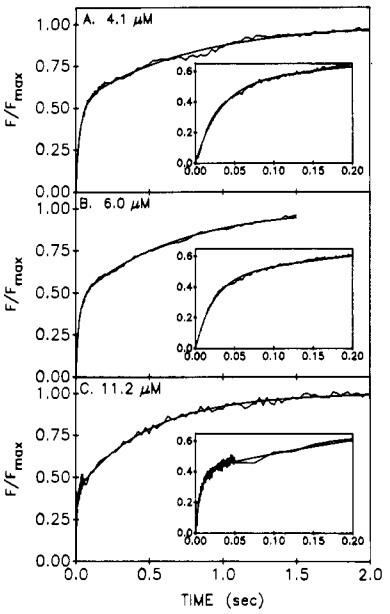


FIGURE 2: Representative data for the kinetics of the reaction of trypsin with $\alpha_2$M under pseudo-first-order conditions, monitored by TNS fluorescence, at several $\alpha_2$M concentrations. The data are expressed as $F/F_{max}$, where $F_{max}$ represents the maximal fluorescence change observed, determined by nonlinear regression analysis of the data to Scheme I. The curves demonstrate the fit of the data to this model. Inset: Expanded scale showing the initial 200 ms of the reaction.

These two distinct phases were readily distinguishable at higher concentrations of $\alpha_2$M, while they were not well resolved at concentrations below 3 $\mu$M, most likely due to the difference in concentration dependence of the two phases (see below). No alterations in the light-scattering properties of the solution during the reaction were noted, suggesting that the slower phase does not represent an aggregation of the $\alpha_2$M–trypsin complex.

*Data Analysis and Formulation of a Model for the Reaction of Trypsin with $\alpha_2$M.* The simplest interpretation of the data assumes that the conformational changes in $\alpha_2$M following reaction with trypsin occur as a set of consecutive reactions, as depicted in Scheme I.

Scheme I

$$\alpha_2M + T \xrightarrow[\Delta F_1]{k_1} \alpha_2M^*\cdot T \xrightarrow[\Delta F_2]{k_2} \alpha_2M^{**}\cdot T$$

In this model, the first step represents the irreversible association of trypsin with $\alpha_2$M giving rise to a portion of the fluorescence change. This step is followed by a second irreversible process that results in an additional signal measured with the probe. The first phase is dependent on reactant concentration, while the second phase is independent of reactant concentration. The data can be fit to the integrated rate equations for Scheme I:

$$C(t) = \frac{k_1BA_0}{k_2 - k_1B}(e^{-k_1Bt} - e^{-k_2t}) \quad (2)$$

and

$$D(t) = A_0\left[1 + \frac{1}{k_1B - k_2}(k_2e^{-k_1Bt} - k_1Be^{-k_2t})\right] \quad (3)$$

where $A_0$ = the initial $\alpha_2$M concentration, $B$ is the concentration of trypsin, $C(t)$ is the concentration of the intermediate complex at time $t$ ($\alpha_2M^*\cdot T$ in Scheme I), and $D(t)$ is the concentration of the final complex at time $t$ ($\alpha_2M^{**}\cdot T$ in Scheme I). The fluorescence signal change observed in converting $\alpha_2$M to $\alpha_2M^*\cdot T$ is $\Delta F_1$, and for converting $\alpha_2M^*\cdot T$ to $\alpha_2M^{**}\cdot T$ is $\Delta F_2$. The total fluorescence at time $t$, $F(t)$, is

$$F(t) = \sum[\Delta F_1C + (\Delta F_1 + \Delta F_2)D] \quad (4)$$

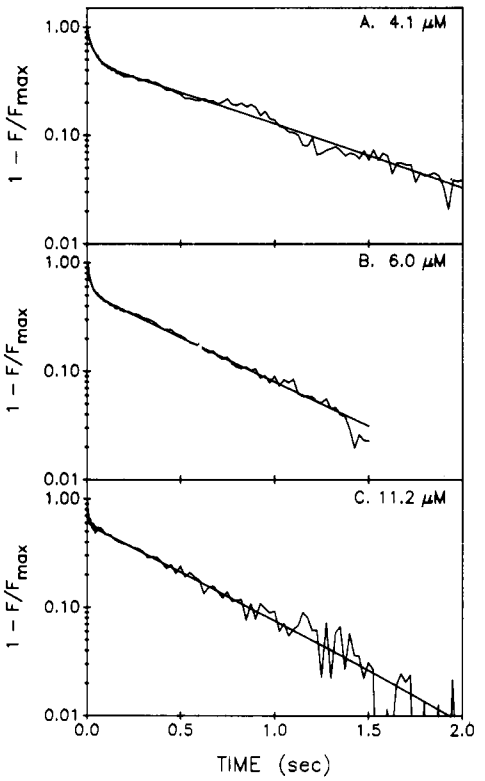


FIGURE 3: First-order plots of the data from the experiments shown in Figure 2. Solid lines represent the best fit of the data to Scheme I using nonlinear regression analysis.

In those instances where a clear distinction between the two phases occurs (i.e., when $[\alpha_2M] > 3$ $\mu$M), it was possible to derive accurate values for the rate constant of each step by fitting each phase individually to a first-order process. A more rigorous fitting procedure on selected data sets was also used in which the entire data sets were fit to Scheme I by nonlinear regression analysis. The smooth curves in Figures 2 and 3 illustrate the best-fit curves obtained by this procedure. Excellent fits of the data to this model were observed, except at low concentrations of $\alpha_2$M (<1.5 $\mu$M), where the fits did not always converge, most likely due to the low signal and high noise level. Consequently, the rate constants derived from these data were not used in the analysis. Using these procedures to determine the values of $k_1$ and $k_2$ at each $\alpha_2$M concentration revealed a linear dependence of the observed rate constant for the first phase of the reaction on $\alpha_2$M concentration (Figure 4). The slope of the best-fit line gave a second-order rate constant of $\sim 1 \times 10^7$ $M^{-1}$ $s^{-1}$. The value of the intercept is close to zero, and cannot be accurately determined from the plot. In contrast, the rate constant of the second phase of the reaction was found to be independent of the concentration of $\alpha_2$M (Figure 5), the data giving an average value of $\sim 1.4$ $s^{-1}$. The best-fit parameters for this model are summarized in Table II.

A model in which the first step of the reaction of $\alpha_2$M with proteinases is reversible (Scheme II) was also considered.

Scheme II

$$\alpha_2M + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \alpha_2M^*\cdot T \xrightarrow[\Delta F_2]{k_2} \alpha_2M^{**}\cdot T$$
$$\Delta F_1$$

Table II. Parameters Derived from Analysis of the Kinetics of Association of $\alpha_2$M with Trypsin in Terms of Schemes I and II[a]

| Scheme I | | | |
|---|---|---|---|
| $k_1$ ($M^{-1}$ $s^{-1}$) | $k_2$ ($s^{-1}$) | $\Delta F_1$[b] | $\Delta F_2$[b] |
| (9.88 ± 0.11) × $10^6$ | 1.44 ± 0.14 | 0.50 ± 0.08 | 0.50 ± 0.08 |

| Scheme II | | | | |
|---|---|---|---|---|
| $k_1$ ($M^{-1}$ $s^{-1}$) | $k_{-1}$ ($s^{-1}$) | $k_2$ ($s^{-1}$) | $\Delta F_1$[b] | $\Delta F_2$[b] |
| (1.0 ± 0.1) × $10^7$ | 0.026 ± 0.04 | 1.84 ± 0.71 | 0.43 ± 0.12 | 0.57 ± 0.12 |

[a] Values are given with their standard deviations determined by nonlinear regression analysis. [b] Fraction of total fluorescence change.

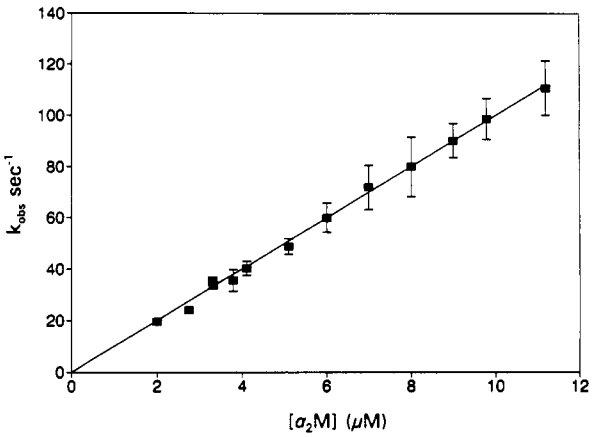


FIGURE 4: Plot of $k_{obs}$ vs the concentration of $\alpha_2$M for the rapid phase of the $\alpha_2$M–trypsin reaction. Each point represents the average of at least six determinations, and the vertical bars show the standard deviations. The line represents the best-fit line determined by linear regression.

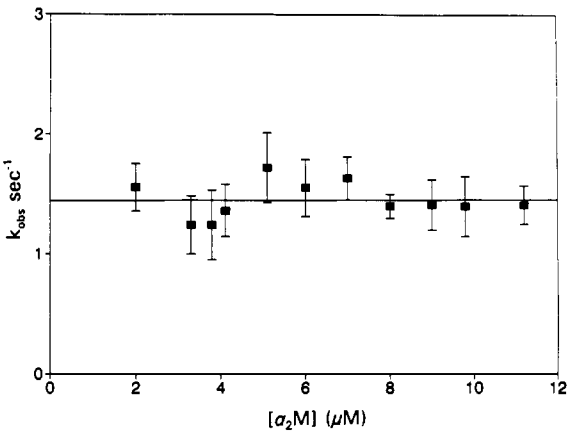


FIGURE 5: Plot of $k_{obs}$ vs the concentration of $\alpha_2$M for the slower phase of the $\alpha_2$M–trypsin reaction. Each point represents the average of at least six determinations, and the vertical bars show the standard deviations. The line represents the best-fit line determined by linear regression.

Following integration of the differential rate equations for this mechanism, it can be shown that

$$C(t) = L_1(e^{-n_1 t} - e^{-n_2 t}) \quad (5)$$

and

$$D(t) = k_2 L_1\left(-\frac{e^{-n_1 t}}{n_1} + \frac{e^{-n_2 t}}{n_2} + \frac{n_2 - n_1}{n_1 n_2}\right) \quad (6)$$

where

$$L_1 = A_0\left(\frac{n_2 - k_1 B}{n_2 - n_1}\right)\left(\frac{k_1 B - n_1}{k_{-1}}\right) \quad (7)$$

and

$$n_1 = \left(\frac{k_1 B + k_2 + k_{-1}}{2}\right)\left(1 - \sqrt{1 - \frac{4(k_1 B k_2)}{(k_1 B + k_2 + k_{-1})^2}}\right) \quad (8)$$

while

$$n_2 = \left(\frac{k_1 B + k_2 + k_{-1}}{2}\right)\left(1 + \sqrt{1 - \frac{4(k_1 B k_2)}{(k_1 B + k_2 + k_{-1})^2}}\right) \quad (9)$$

where $A_0$ = the initial $\alpha_2$M concentration, $B$ is the concentration of trypsin, $C(t)$ is the concentration of the intermediate complex at time $t$ ($\alpha_2$M*·T in Scheme II), and $D(t)$ is the concentration of the final complex at time $t$ ($\alpha_2$M**·T in Scheme II).

The data were fit to this mechanism by nonlinear regression analysis. An excellent fit of the data was obtained at all but low (i.e., <1.5 $\mu$M) concentrations of $\alpha_2$M, and, in general, it was impossible to discriminate between Scheme I and Scheme II. The best-fit results derived for this model are summarized in Table II. Examination of the results reveals that the association rate constants are essentially identical with those obtained in Scheme I. Moreover, if a reversible step does occur, it is very slow, and the binding is of high affinity, as shown by the dissociation constant of 2.6 nM calculated for the initial step from the rate constants (i.e., $K_d = k_{-1}/k_1$).

Additional, more complex models, such as that depicted in Scheme III, were considered. A mechanism similar to this one has been shown to adequately describe the association of several serine proteinases with their inhibitors (Laskowski & Kato, 1980).

Scheme III

$$\alpha_2M + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \alpha_2M{\cdot}T \xrightarrow[\Delta F_1]{k_2} \alpha_2M^*{\cdot}T \xrightarrow[\Delta F_2]{k_3} \alpha_2M^{**}{\cdot}T$$

In this model, the association of trypsin with $\alpha_2$M is assumed to involve an initial binding interaction or Michaelis complex, $\alpha_2$M·T, before the irreversible formation of an intermediate complex, $\alpha_2$M*·T, giving rise to a change in fluorescence, $\Delta F_1$. This complex then undergoes an additional conformational change to the final state, $\alpha_2$M**·T, resulting in additional fluorescence alterations, $\Delta F_2$. While direct fitting of the data to this mechanism was attempted, in most data sets the fit did not converge, most likely due to the complexity of the model. This mechanism predicts a hyperbolic dependence of $k_{obs}$ on $\alpha_2$M concentration for the rapid phase. The linear concentration dependence observed in this study thus would suggest that Scheme I or II more accurately describes the reaction mechanism. However, it is possible that the hyperbolic dependence of $k_{obs}$ may only be observed at concentrations of $\alpha_2$M that greatly exceed those obtainable with this protein.

## Discussion

Two previous studies have used rapid-kinetic techniques to characterize the reaction of trypsin (Dangott et al., 1983) or chymotrypsin (Favaudon et al., 1987) with $\alpha_2$M. An initial, preliminary study (Dangott et al., 1983), performed at a 2-fold

molar excess of trypsin over $\alpha_2$M ([$\alpha_2$M] = 1 $\mu$M) and using tryptophan fluorescence to monitor the reaction, observed biphasic kinetics. The second study (Favaudon et al., 1987) measured changes in TNS fluorescence, in tryptophan fluorescence, and in the fluorescence of fluorescein isothiocyanate labeled chymotrypsin to monitor the reaction at various molar ratios of enzyme to $\alpha_2$M. In all cases, the data were fit to the sum of three exponentials, indicative of the reaction occurring in three successive first-order steps. While both of these studies substantiate the complexity of the reaction of proteinases with $\alpha_2$M, neither of the investigations examined the effect of varying the reactant concentration under pseudo-first-order conditions, or analyzed the reaction under conditions to minimize proteolysis at secondary sites other than those found within the "bait" region. Consequently, it is difficult to interpret the data in terms of an appropriate mechanism.

In the present investigation, the experiments were performed under conditions of $\alpha_2$M excess, where essentially only 1:1 $\alpha_2$M–trypsin complexes are formed, so that the kinetics reflect the binding of the proteinase to only one binding site. Moreover, the risk of cleavage of the complex at secondary sites by excess proteinase is greatly reduced. To monitor the reaction, the fluorescent dye TNS was chosen, since previous studies have shown this probe to be sensitive to conformational changes accompanying complex formation of $\alpha_2$M with proteinases. The available evidence suggests that this dye is monitoring events occurring within the $\alpha_2$M molecule. First of all, the titration data could be fit to a single class of sites giving different dissociation constants for $\alpha_2$M and $\alpha_2$M–trypsin, suggesting that the dye binds weakly to native $\alpha_2$M and that conversion of native to proteolyzed $\alpha_2$M primarily results in an increased affinity of the protein for the dye. Second, only a weak interaction between trypsin and the dye was shown to occur, consistent with the observation that $\alpha_2$M previously reacted with methylamine has similar dye binding properties as the $\alpha_2$M–trypsin complex (Strickland & Bhattacharya, 1984).

The kinetic measurements showed that the reaction of trypsin with $\alpha_2$M, monitored with TNS as a probe under conditions of $\alpha_2$M excess, can be resolved into two distinct steps. The first of these, accounting for approximately half of the fluorescence signal, is a second-order process, showing a linear dependence of the observed pseudo-first-order rate constant on reactant concentration. The subsequent step, giving rise to the remaining half of the fluorescence signal, is a first-order reaction with an observed rate constant independent of reactant concentration. The simplest mechanism satisfactorily accounting for these data is a sequential reaction described by Scheme I. This mechanism predicts an initial rapid association of trypsin with $\alpha_2$M with a rate constant of $1.0 \times 10^7$ $M^{-1}$ $s^{-1}$. This value is close to that of $2 \times 10^7$ $M^{-1}$ $s^{-1}$ estimated for the $\alpha_2$M–trypsin reaction from analyses of the rate of thiol ester bond cleavage under conditions of $\alpha_2$M excess, but in the presence of high concentrations of benzamidine to slow the reaction down (Christensen & Sottrup-Jensen, 1984). A corresponding second-order step was apparently not observed in the stopped-flow analyses of the reaction of $\alpha_2$M with chymotrypsin (Favaudon et al., 1987). The reaction scheme further suggests that the initial rapid association is followed by a slower conformational change of $\alpha_2$M with a rate constant of $\sim$1.4 $s^{-1}$. Even this slow step is considerably faster than any of the three first-order steps reported for the $\alpha_2$M–chymotrypsin reaction, the fastest of which had a rate constant of $\sim$0.3 $s^{-1}$ (Favaudon et al., 1987).

The assignment of two phases of the $\alpha_2$M–trypsin reaction to individual molecular steps of this reaction remains tentative at this time. A definitive assignment will require more sophisticated techniques such as rapid quenched-flow analyses evaluated by parallel measurements of peptide bond cleavage, liberated sulfhydryl groups, and conformational changes. Nevertheless, the second-order nature and the magnitude of the rate constant of the first step suggest that this step reflects cleavage of the $\alpha_2$M bait region by the proteinase. This cleavage most likely results in altered mobility of the polypeptide chain in the vicinity of the cleavage site, which could result in an increased fluorescence of the bound probe. Alternatively, the TNS fluorescence change of the first step may reflect an intramolecular reaction that occurs subsequent to bait region cleavage with a sufficiently high rate constant that cannot be kinetically distinguished from this cleavage under the conditions investigated. A possible candidate for such a reaction is the activation of the thiol ester bonds, which may be accompanied by altered TNS binding due to local structural perturbations. This possibility is supported by the report that bait region cleavage, as studied by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and thiol ester bond cleavage, as reflected in the appearance of free sulfhydryl groups, occurred with comparable rates in studies of the $\alpha_2$M–trypsin reaction in the presence of benzamidine (Christensen & Sottrup-Jensen, 1984). It is furthermore possible that the conformational change proposed to be involved in noncovalent binding ("trapping") of the enzyme to the inhibitor (Barrett & Starkey, 1973; Gonias et al., 1982; Björk & Fish, 1982), a change that also could affect TNS binding, has such a high rate constant that this rate is indistinguishable from that of the rate-limiting bait region cleavage under the conditions used.

The slow step demonstrated by the kinetic analysis with TNS as a probe must reflect an intramolecular conformational rearrangement of $\alpha_2$M occurring subsequent to bait region cleavage. It is unlikely that this conformational change is responsible for the noncovalent binding ("trapping") of the enzyme, since the slow rate would be expected to lead to inefficient trapping. This would be in contrast to experimental observations indicating that the trapping efficiency is extremely high for the first molecule of proteinase bound (Larsson et al., 1989). Instead, the magnitude of this rate suggests a more plausible alternative. The model presented by Larsson et al. (1989), which is supported by other work (Howell et al., 1983; Salvesen et al., 1983; Christensen & Sottrup-Jensen, 1984; Björk, 1984; Pochon, 1987), thus proposes that once an enzyme molecule reacts with one of the two binding sites in $\alpha_2$M, an intermediate complex forms that either can react with a second proteinase molecule or can undergo a slow conformational rearrangement to yield a complex that is largely unable to bind additional proteinase. Modeling experiments have suggested that the magnitude of the rate constant for this conformational change is 1–2 $s^{-1}$ (Larsson et al., 1989), values which are close to that of 1.4 $s^{-1}$ measured for the slow phase in the present investigation. This phase therefore may reflect the putative conformational rearrangement of $\alpha_2$M that leads to decay of the second proteinase binding site (Björk, 1984; Larsson et al., 1989). The data in the present study thus provide the first direct kinetic evidence supporting the proposed model.

**Registry No.** T, 9002-07-7; proteinase, 9001-92-7.

## References

Ashcom, J. D., Tiller, S. E., Dickerson, K., Cravens, J. L., Argraves, W. S., & Strickland, D. K. (1990) *J. Cell Biol. 110*, 1041–1048.

Barrett, A. J. (1981) *Methods Enzymol. 80*, 737–754.
Barrett, A. J., & Starkey, P. M. (1973) *Biochem. J. 133*, 709–724.
Björk, I. (1984) *Biochem. Biophys. Res. Commun. 118*, 691–695.
Björk, I., & Fish, W. W. (1982) *Biochem. J. 207*, 347–356.
Björk, I., Larsson, L. J., Lindblom, T., & Raub, E. (1984) *Biochem. J. 217*, 303–308.
Björk, I., Lindblom, T., & Lindahl, P. (1985) *Biochemistry 24*, 2653–2660.
Björk, I., Alriksson, E., & Ylinenjärvi, K. (1989) *Biochemistry 28*, 1568–1573.
Chase, T., & Shaw, E. (1970) *Methods Enzymol. 19*, 20–27.
Christensen, U., & Sottrup-Jensen, L. (1984) *Biochemistry 23*, 6619–6626.
Dangott, L. J., Puett, D., & Cunningham, L. W. (1983) *Biochemistry 22*, 3647–3653.
Favaudon, V., Tourbez, M., Pochon, F., Mareix, R., & Tourbez, H. (1987) *Eur. J. Biochem. 165*, 31–37.
Ganrot, P. O. (1966) *Acta Chem. Scand. 20*, 2299–2300.
Gonias, S. L., & Pizzo, S. V. (1983) *Biochemistry 22*, 536–546.
Gonias, S. L., Reynolds, J. A., & Pizzo, S. V. (1982) *Biochim. Biophys. Acta 705*, 306–314.
Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 171*, 27–38.
Harpel, P. C. (1973) *J. Exp. Med. 138*, 508–521.
Howell, J. B., Beck, T., Bates, B., & Hunter, M. J. (1983) *Arch. Biochem. Biophys. 221*, 261–270.
Imber, M. J., & Pizzo, S. V. (1981) *J. Biol. Chem. 256*, 8134–8139.
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*, 7323–7328.
Larsson, L.-J., Frisch, E. P., Törneke, K., Lindblom, T., & Björk, I. (1988) *Thromb. Res. 49*, 55–68.
Larsson, L.-J., Neuenschwander, D. E., & Strickland, D. K. (1989) *Biochemistry 28*, 7636–7643.
Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593–626.
Liu, D., Feinman, R. D., & Wang, D. (1987) *Biochemistry 26*, 5221–5226.
McClure, W. O., & Edelman, G. M. (1966) *Biochemistry 5*, 1908–1918.
McClure, W. O., & Edelman, G. M. (1967) *Biochemistry 6*, 559–566.
Moestrup, S. K., & Gliemann, J. (1989) *J. Biol. Chem. 264*, 15574–15577.
Pochon, F. (1987) *Biochim. Biophys. Acta 995*, 37–45.
Robinson, N. C., Tye, R. W., Neurath, H., & Walsh, K. A. (1971) *Biochemistry 10*, 2743–2747.
Salvesen, G., Virca, G. D., & Travis, J. (1983) *Ann. N.Y. Acad. Sci. 421*, 316–326.
Sottrup-Jensen, L., & Birkedal-Hansen, H. (1989) *J. Biol. Chem. 264*, 393–401.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981) *FEBS Lett. 128*, 123–126.
Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Wierzbicki, D. M., Jones, C. M., Lønblad, P. B., Magnusson, S., & Petersen, T. E. (1984) *J. Biol. Chem. 259*, 8318–8327.
Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Lønblad, P. B., Jones, C. M., Wierzbicki, D. M., Magnusson, S., Domdey, H., Wetsel, R. A., Lundwall, Å., Tack, B. F., & Fey, G. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 9–13.
Steiner, J. P., Bhattacharya, P., & Strickland, D. K. (1985) *Biochemistry 24*, 2993–3001.
Steiner, J. P., Migliorini, M., & Strickland, D. K. (1987) *Biochemistry 26*, 8487–8495.
Strickland, D. K., & Bhattacharya (1984) *Biochemistry 23*, 3115.
Strickland, D. K., Steiner, J. P., Feldman, S. R., & Pizzo, S. V. (1984) *Biochemistry 23*, 6679–6685.
Strickland, D. K., Steiner, J. P., Migliorini, M., & Battey, F. D. (1988) *Biochemistry 27*, 1458–1466.
Strickland, D. K., Ashcom, J. D., Williams, S., Burghess, W. H., & Argraves, W. S. (1990) *J. Biol. Chem. 265*, 17401–17404.
Swenson, R. P., & Howard, J. B. (1979) *J. Biol. Chem. 254*, 4452–4456.
Walsh, K. A., & Neurath, H. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 884–889.
Yung, B. Y., & Trowbridge, C. G. (1975) *Biochem. Biophys. Res. Commun. 65*, 927–930.