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Intrinsic fluorescence changes and rapid kinetics of proteinase deformation during serpin inhibition

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Abstract The X-ray crystal structure of the serpin–proteinase complex suggested that the serpin deformed the proteinase thereby inactivating the molecule. Using a variant of α_1-antitrypsin in which both tryptophan residues have been replaced by phenylalanine, we have shown that the proteinase becomes partially unfolded during serpin inhibition. The tryptophan free variant, α_1-antitrypsin$_{(FF)}$, is fully active as an inhibitor of thrombin. Thrombin has a fluorescence emission maximum of 340 nm which blue shifts to 346 nm, concomitant with a 40% increase in intensity, upon formation of the serpin–proteinase complex indicative of substantial conformational change within the proteinase. Stopped-flow analysis of the fluorescence changes within the proteinase indicated a two-step mechanism. A fast bimolecular reaction with a rate constant of 2.8×10^6 M^{-1} s^{-1} is followed by a slow unimolecular process with a rate of 0.26 s^{-1} that is independent of concentration. We propose that the first rate is formation of an initial complex which is then followed by a slower process involving the partial unfolding of the proteinase during its translocation to the opposite pole of the serpin.

Key words: Serpin; Proteinase; Protein folding; Conformational change

1. Introduction

The serpin (serine proteinase inhibitor) superfamily of proteins control a wide range of physiological events such as coagulation, complement activation and apoptosis [1]. While many serpins function primarily as serpins it has recently been demonstrated that some are also inhibitors of cysteine proteinases [2,3]. The crystal structures of a number of serpins are known and they all conform to a standard architecture consisting of a core of three β-sheets surrounded by nine α-helices [4–10]. Protruding from the A β-sheet is the reactive center loop, a stretch of amino acid residues which contains the scissile bond. The primary sequence of this region plays the major role in determining serpin inhibitory specificity [11].

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Abbreviations: α_1-AT, α_1-antitrypsin; EI*, final serpin proteinase complex; EI_M, initial Michaelis-type complex; λ_{ex}, excitation wavelength; λ_{max}, emission maximum; serpin, serine proteinase inhibitor

The simplest kinetic scheme which describes the serpin proteinase interaction is:

$$E + I \overset{K_{-1}}{\leftrightarrow} EI_M \xrightarrow{k_2} EI^* \qquad \text{Scheme 1}$$

Where K_{-1} is the equilibrium dissociation constant for the Michaelis-type complex and k_2 is the rate constant for the pseudo-irreversible or irreversible transformation of EI_M into EI*, the final inhibited complex.

The nature of the serpin-specific conformational changes involved in each of these steps is controversial. Ourselves and others have shown that formation of EI_M involves partial insertion of the reactive center loop into the A β-sheet of the serpin [12,13], whereas Gettins and co-workers have recently shown that nuclear magnetic resonance (NMR) data suggest that no change occurs in the serpin [14]. The transformation from EI_M to EI*, which probably involves a number of steps, results in the translocation of the proteinase to the opposite pole of the molecule. The recently solved crystal structure of a serpin–proteinase complex clearly demonstrates that the reactive center loop is fully inserted into the A β-sheet [10], consistent with this movement. Two studies using biophysical techniques and epitope mapping, however, contrast with these structural data and suggest that the final complex is an ensemble of conformations in which the reactive center loop is in an equilibrium between partial and full insertion [15,16]. It is the inherent instability of the serpin molecule that drives this substantial conformational change [17], such that proteinase inhibition results in a marked increase in the stability of the serpin [18]. The ability to undergo such conformational changes also makes the serpin molecule extremely susceptible to mutations that result in inappropriate conformational changes which can cause diseases such as emphysema, thrombosis and liver disease [19,20].

Whilst the conformational changes within the serpin have been intensively studied, little work has focused on proteinase conformational change. ^{1}H NMR studies have indicated that conformational changes occur in the proteinase active site [18,21]. It has also been demonstrated that the proteolytic vulnerability of certain loops within the proteinase is increased in the serpin–enzyme complex compared with the uninhibited state, suggesting conformational change [18,22,23]. The crystal structure of the serpin–proteinase complex confirmed these findings, indicating that the proteinase had become partially disordered, based upon the lack of electron density for 37% of the structure [10].

In order to characterize the proteinase conformational change further we have utilized fluorescence spectroscopy,

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which is an extremely sensitive technique that can be used to follow both protein folding and binding events. Interpretation of fluorescence data is difficult, however, if both proteins possess tryptophan residues, as the origin of any signal change is unclear. We have replaced the two tryptophan residues of α_1-antitrypsin (α_1-AT) with phenylalanine to create a fluorescently silent variant of α_1-AT that is fully active as an inhibitor. This has allowed for the first time direct observation of the conformational changes in the proteinase during serpin inhibition. Our data strongly suggest a two-step mechanism in which substantial proteinase unfolding occurs during the transformation of EI_M to EI*.

2. Materials and methods

2.1. Production and characterization of α_1-$AT_{(FF)}$

In this study we used the Pittsburgh variant (where the P1 = Arg) of α_1-AT as our template for mutagenesis. α_1-$AT_{(P1=Arg)}$ contains two tryptophan residues numbered 194 and 238, which were replaced by phenylalanine residues using the Quick change site-directed mutagenesis kit (Stratagene) to produce α_1-$AT_{(FF)}$. The mutations were verified using DNA sequencing. α_1-$AT_{(FF)}$ was expressed in *Escherichia coli* as inclusion bodies and purified as previously described [24]. The association rate constant (k_{ass}) and stoichiometry of inhibition (SI) for the interaction of α_1-$AT_{(FF)}$ with thrombin were determined as described previously [25].

2.2. Production and characterization of thrombin

Thrombin was purified from human plasma and characterized as previously described [26].

2.3. Spectroscopic experiments

All fluorescence experiments were performed in a 50 mM Tris, pH 7.8, 0.1 M NaCl buffer at 25°C using a Perkin Elmer LS50B spectrofluorimeter. Emission spectra were recorded between 300 and 400 nm (λ_{ex} = 280 nm) at scan rates of 1 nm/s with slit widths of 2.5 nm for both excitation and emission.

2.4. Circular dichroism (CD)

CD experiments were performed on a Jasco 820s spectropolarimeter using a protein concentration of 0.05 mg/ml in 50 mM Tris, 100 mM NaCl, pH 7.8. Thermal unfolding was performed using a heating rate of 60°C/h and the changes in secondary structure with temperature were measured by monitoring the CD signal at 222 nm.

2.5. Kinetic experiments

All kinetic experiments were performed using an Applied Photophysics SF.18MV stopped-flow apparatus. The proteinase conformational change was monitored by following changes in fluorescence intensity (λ_{ex} = 290 nm) at wavelengths > 320 nm using a cut-off filter placed between the reaction cell and the detector. Equal volumes of proteinase and serpin were rapidly mixed at 25°C under pseudo-first order conditions and the reaction followed. Both solutions contained 50 mM Tris, 0.1 M NaCl, pH 7.8. Data collected from at least eight traces were averaged and fitted to either a single or double exponential function using the manufacturer's software.

3. Results

The recent structure of the serpin–proteinase complex provided direct structural evidence that during inhibition the proteinase becomes partially distorted [10]. The aim of this work was to determine in solution the extent and kinetics of this conformational change using fluorescence spectroscopy. To achieve this we created a fluorescently silent α_1-AT variant (α_1-$AT_{(FF)}$) in which both native tryptophan residues (194 and 238) were replaced by phenylalanine. We chose the Pittsburgh variant of α_1-AT for these studies as numerous studies have shown that this serpin variant functions as a classic serpin, inhibiting thrombin with high efficiency [12,14,27]. Due to the replacement of both tryptophan residues (in particular Trp-194 which is conserved within 94% of the serpin superfamily [28]) we characterized the stability and activity of α_1-$AT_{(FF)}$. The far-UV CD spectrum of α_1-$AT_{(FF)}$ was identical to that of wild-type antitrypsin (data not shown), indicating that the mutations had not significantly altered the serpin's structure and that the tryptophan residues did not contribute to the far-UV spectrum. The stability of the serpin was assessed using a thermal melt yielding a melting temperature of 58°C, similar to that of the wild-type (Table 1). α_1-$AT_{(FF)}$ displayed a SI of 1.2, which was confirmed by the presence of a small amount of cleaved material seen in sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of the complex (Table 1; insert to Fig. 1) and a k_{ass} of 6×10^5 M^{-1} s^{-1} with thrombin, indicating that the double mutation had no significant effect upon the inhibitory characteristics of the protein (Table 1).

Table 1

Protein	$k_{ass}\times10^5$ (M^{-1} s^{-1})	SI	T_M (°C)
α_1-$AT_{(P1=Arg)}$	4.9 ± 02	1.1	59.6 ± 0.3
α_1-$AT_{(FF)}$	6.7 ± 0.15	1.2	57.9 ± 0.2

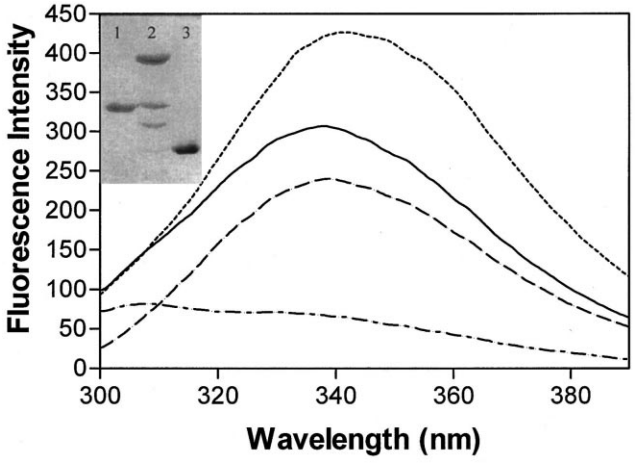

Fig. 1. Fluorescence emission spectra of thrombin and thrombin–α_1-$AT_{(FF)}$ complex. The fluorescence emission spectra of 200 nM thrombin (dashed line), 200 nM α_1-$AT_{(FF)}$ (dashed and dots), the sum of thrombin and α_1-$AT_{(FF)}$ spectra (solid line) and the thrombin–α_1-$AT_{(FF)}$ complex (dashed line) are shown. The excitation wavelength was 290 nm with a bandpass of 2.5 nm and a temperature of 25°C. Each spectrum represents the average of five scans and two different protein preparations. The inset shows the proteins used for the spectra: 1 – α_1-$AT_{(FF)}$; 2 – the complex between α_1-$AT_{(FF)}$ and thrombin and 3 – thrombin.

The phenylalanine substitutions, however, had dramatic effects upon the emission spectra of α_1-$AT_{(FF)}$ (Fig. 1). α_1-AT normally has an emission maximum at 337 nm (λ_{ex} = 280 nm), indicative of its two partially buried tryptophan residues, whereas α_1-$AT_{(FF)}$ possesses an emission maximum of 308 nm (λ_{ex} = 280 nm), indicative of a protein totally devoid of tryptophan residues (Fig. 1). The native state of thrombin has an emission maximum at 340 nm, which represents a global picture of the nine tryptophan residues in the thrombin molecule. Upon addition of equimolar amounts of α_1-$AT_{(FF)}$ to thrombin, the tryptophan emission maximum became red-shifted to 346 nm concomitant with a 40% increase in fluo-

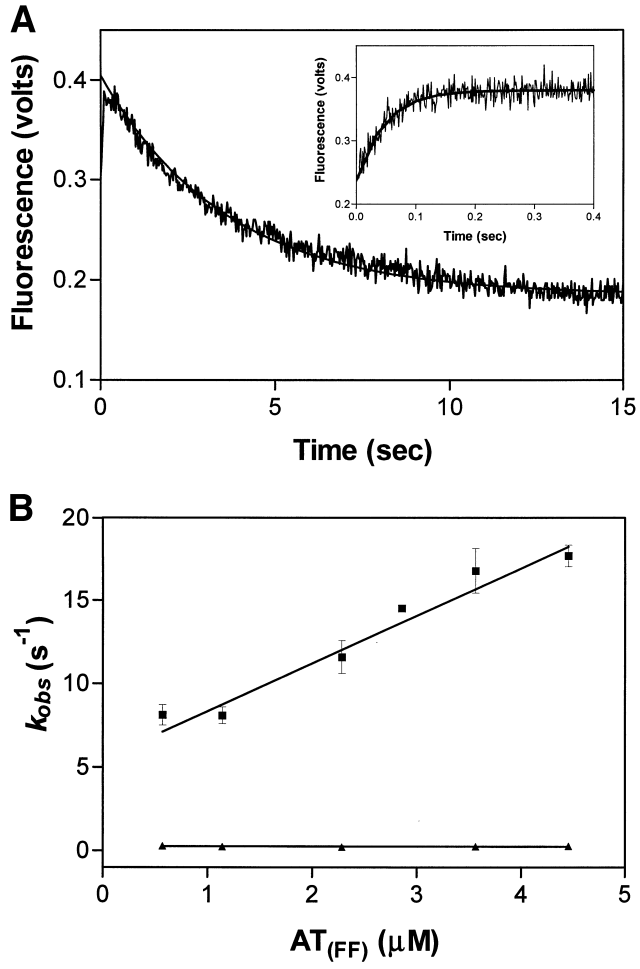

Fig. 2. Reaction of thrombin with α_1-$AT_{(FF)}$. (A) Thrombin (0.1 μM) and α_1-$AT_{(FF)}$ (4.5 μM) were rapidly mixed and the change in fluorescence followed. The inset shows the initial increase in fluorescence occurring over the first 400 ms. The data were fitted to a single exponential function to yield a value of 18.4 ± 0.3 s^{-1}. This is followed by a decrease in intensity which was fitted to a single exponential decay to yield a value of 0.24 ± 0.02 s^{-1}. (B) Dependence of the values for k_{obs} on the concentration of α_1-$AT_{(FF)}$. Analysis of the data similar to those shown in A yielded estimates for k_{obs1} (■) and k_{obs2} (▲); these are plotted together with their standard errors. The data for k_{obs1} were fitted to Eq. 1 and the line shows the results of this analysis.

rescence intensity. These changes are indicative of the tryptophan residues of thrombin becoming more solvent-exposed, suggesting partial unfolding of the proteinase following inhibition.

In order to further characterize this conformational change we undertook stopped-flow fluorescence studies to determine the individual rate constants for the process. α_1-$AT_{(FF)}$ and thrombin were rapidly mixed under pseudo-first order conditions in which α_1-$AT_{(FF)}$ was at a minimum of 7-fold molar excess. A typical reaction progress curve obtained after mixing is shown in Fig. 2A. The curve shows a rapid increase in fluorescence intensity with a half-life of $\approx$30 ms followed by a decrease in fluorescence intensity with a half-life of $\approx$3.5 s. Analysis of the data obtained between 0.5 and 4.5 μM yielded both the rate constants (k_{obs1} and k_{obs2}) and the amplitudes for the two phases; the values obtained for the rate constants are shown in Fig. 2B. The amplitudes of the two phases did not vary significantly with the concentration of α_1-$AT_{(FF)}$, the mean amplitudes of the first and second phases were +0.09 and −0.18, respectively. Fig. 2B shows the dependence of the observed rate constants on the concentration of α_1-$AT_{(FF)}$. The rate of initial increase in fluorescence (k_{obs1}) was found to be linearly dependent on the concentration of α_1-$AT_{(FF)}$, which is typical of a simple bimolecular interaction. Analysis of the variation in k_{obs1} according to

$$k_{obs} = k_1[I] + k_{-1} \quad (1)$$

[29] where k_1 is the apparent second order rate constant for the formation of the initial serpin enzyme complex yields a value of $2.8 \pm 0.2 \times 10^6$ M^{-1} s^{-1}. The concentration dependence of k_{obs1} indicates that it is following conformational changes within thrombin due to its binding to the serpin and formation of EI_M. The dissociation rate constant of the initial complex, k_{-1}, was determined to be 5.5 ± 0.3 s^{-1}. The equilibrium dissociation constant K_{-1} of the EI_M complex, calculated from k_1 and k_{-1}, was 0.51 μM.

k_{obs2}, which describes the large decrease in fluorescence intensity following formation of the docking complex, did not vary significantly between 0.5 and 4.5 μM α_1-$AT_{(FF)}$. The lack of variation in concentration dependency of k_{obs2} (weighted mean for k_{obs2} was 0.26 ± 0.01 s^{-1}) indicates that the transformation of EI_M to the final complex is a first order, irreversible process. We propose that k_2 reports a slow conformational change within the proteinase, following formation of EI_M, which leads to complete inhibition of the proteinase.

4. Discussion

4.1. Characterization of α_1-$AT_{(FF)}$

Throughout this study we have used the double tryptophan knockout α_1-$AT_{(FF)}$ in which both Trp-194 and -238 have been replaced. α_1-$AT_{(FF)}$ was found to be as stable as α_1-$AT_{(P1=Arg)}$ and fully active against thrombin. This result is significant because Trp-194, which is situated at the top of the A β-sheet on strand 3A, is one of the most highly conserved residues (94%) across the serpin superfamily [28]. Therefore, the double replacement results in a molecule without fluorescence in the tryptophan region of the spectrum that has retained full activity and stability.

4.2. Interpretation of the fluorescence changes

Using a fluorescently silent serpin we have begun to directly probe the proteinase conformational changes that occur during inhibition. Stopped-flow analysis of the reaction identified two phases, the first a rapid enhancement in fluorescence intensity followed by a slower decrease. Thrombin possesses nine tryptophan residues spread throughout the molecule and the enhancement in fluorescence could be caused by a change in exposure of one or more of them. It has previously been shown that the intrinsic fluorescence of thrombin is increased when it binds hirudin and other active site-specific inhibitors [30,31]. Analysis of the crystal structure of various thrombin–inhibitor complexes and thermodynamic data suggests that the environment of three tryptophan residues would be significantly changed [32,33]. These residues Trp-60, Trp-96 and Trp-148 are all situated on flexible loops that surround the active site. It has been proposed from the structure of thrombin with BPTI that Trp-60 and Trp-148 on the 60-

and 148-loops would form stabilizing interactions with a serpin during formation of the docking complex [32]. The initial enhancement in fluorescence is concentration dependent (Fig. 2B), which suggests a bimolecular process. Given the previous fluorescence data described above and our own we suggest that the first step is formation of the initial docking complex (EI_M) which requires significant movement of thrombin's loops.

The second fluorescence change is a decrease in intensity. This effect is strongly suggestive of increased solvent exposure of one or more of the tryptophan residues within thrombin. Given the magnitude of the change and the large number of tryptophan residues involved it is difficult to assign any specific structural changes.

Stopped-flow analysis of the reaction between α_1-$AT_{(FF)}$ and thrombin has enabled us to directly monitor the conformational changes within thrombin as it becomes inhibited. Our data show that within less than 1 s after mixing an initial complex EI_M (which has increased fluorescence intensity) is formed in a second order process. The rate constants characterizing the formation and breakdown of EI_M are 2.8×10^6 M^{-1} s^{-1} and 5.5 s^{-1}, respectively. These values agree extremely well with those determined by others [34]. This also indicates that the use of α_1-$AT_{(FF)}$ and thrombin represents a good model for examining the serpin–proteinase interaction. The second step of the reaction is a first order process that results in a large decrease in fluorescence. k_2 (0.2 s^{-1}) represents the rate of conversion of EI_M to EI*. This value also agrees well with the rate of translocation of the proteinase from one pole of the molecule to another as observed by others (0.13 s^{-1} [34]).

This study follows the conformational changes observed solely in the proteinase for the first time. As the deformation of the proteinase occurs on the same time scale as translocation we propose that the proteinase becomes distorted during its journey from one end of the serpin to the other. These findings support the hypothesis generated from the recent serpin–proteinase crystal structure in which it is proposed that the proteinase is 'crushed' against the body of the serpin as it moves towards its final position [10]. This process is driven by the ability of the serpin to rapidly insert its reactive center loop residues into the body of the serpin. Interestingly recent work has shown that the rate of insertion due to inappropriate cleavage of the reactive center loop is approximately 10-fold faster than when a proteinase is attached [35]. Carrell and colleagues propose that the conformational change in the proteinase is required for by-passing the F-helix which lies across the A β-sheet [10]: here we provide direct spectroscopic data to support this.

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