The spontaneous polymerization of plasminogen activator inhibitor type-2 and Z-antitrypsin are due to different molecular aberrations

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Abstract The wild-type form of plasminogen activator inhibitor type-2 (PAI-2) and the pathogenic Z-mutant of α_1-antitrypsin (α_1AT) are serpins that spontaneously polymerize by the loop–sheet mechanism. Compared to the consensus serpin sequence, both PAI-2 and Z-α_1AT have deviations in the so-called breach region located at the top of the A β-sheet. In the case of Z-α_1AT, conformational perturbations caused by a single amino acid substitution result in polymerization in vivo and predisposes to disease. To test whether the polymerization of PAI-2 is due to aberrations in the breach region, we constructed substitution mutants of PAI-2 with conserved residues in this region. Analysis of the mutants revealed that deviations in the breach region modulate but are not the major cause of PAI-2 polymerization. Rather, PAI-2 exists in a highly polymerogenic conformation and does not require conformational rearrangements before polymerization can take place.

Key words: Serpin; Loop–sheet polymerization; Plasminogen activator inhibitor type-2

1. Introduction

Plasminogen activator inhibitor type-2 (PAI-2) is a member of the large and diverse serpin family that encompasses a wide range of proteins, mostly protease inhibitors [1–3]. The members of this protein family have a common molecular architecture based on a dominant five-stranded A β-sheet surrounded by β-sheets B and C, and a mobile reactive center loop (RCL) located above the core of the molecule [4–7]. Serpin inhibition involves reactive center cleavage and insertion of the RCL into the A β-sheet, whereby the covalently linked protease is translocated from one pole of the molecule to the opposite one [8–14].

In their active forms, the inhibitory serpins are metastable molecules that readily convert to more stable forms when the RCL inserts into the A β-sheet, e.g. during complex formation or when so-called latent or cleaved species are formed [15–19]. The metastability of the serpins also accounts for the formation of an additional conformational state that involves unique intermolecular linkages between serpin molecules, whereby so-called loop–sheet polymers are formed. The polymerization proceeds by a sequential insertion of the RCL from one serpin molecule into the open A β-sheet of another and is accompanied by a loss of serpin inhibitory activity [20–24]. Native wild-type (wt) serpins are rather stable and the polymerization can be induced only after exposure to mild denaturing conditions. However, some naturally occurring pathological mutations perturb the serpin structure so that polymers are formed even under physiological conditions [25]. The spontaneously polymerizing Z-mutant of α_1-antitrypsin (α_1AT) has been extensively studied because it accumulates as polymers in hepatocytes, leading to cirrhosis and emphysema [23]. Z-α_1AT differs from the wt inhibitor by a single substitution of E342 to K, that is located in the breach region where loop insertion is initiated (Fig. 1A,B) [26]. The mutation disrupts a salt bridge and induces molecular perturbations which cause an opening of the A β-sheet, resulting in a structure that is prone to polymerize [21]. It is not clear whether polymerization of the Z-α_1AT is the cause of secretory block of this mutant, or, alternatively, whether the polymerization is the result of an inefficient secretion. Although it was proposed that the secretory block of Z-α_1AT is caused by disruption of a conserved salt bridge between K290 and E342 [15], it seems more likely now that it is not the salt bridge, but rather a negatively charged amino acid at the 342 position which is crucial for the secretion of α_1AT [27–29].

PAI-2 is synthesized in both extracellular and intracellular forms [30–32]. In the extracellular compartment PAI-2 inhibits urokinase-type plasminogen activator (uPA), but so far no intracellular target protease has been identified. However, intracellular PAI-2 has been associated with cell protection against apoptosis [33,34] and keratinocyte differentiation [35]. An unusual feature of PAI-2 is that the normal wt form of this serpin spontaneously forms loop–sheet polymers under physiological conditions [36–38]. The polymerization is concentration dependent and may occur both in vitro and in vivo. The molecular determinants of PAI-2 polymerization are unknown but, like the Z-α_1AT, PAI-2 has deviations in the breach region (Fig. 1C), which may influence PAI-2's ability to polymerize. To search for the molecular determinants causing PAI-2 polymerization, we have studied substitution mutants of PAI-2 with a conserved molecular arrangement in the breach region.

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Abbreviations: α_1AT, α_1-antitrypsin; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; PAGE, polyacrylamide gel electrophoresis; PAI-2, plasminogen activator inhibitor type 2; RCL, reactive center loop; s3A, β-strand 3 of A β-sheet; uPA, urokinase-type plasminogen activator; wt, wild-type

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2. Materials and methods

2.1. Construction of PAI-2 mutants for expression in prokaryotic system

Mutagenesis was performed using Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and was followed by full-length cDNA sequencing. PAI-2 was expressed in the *Escherichia coli* strain AD494(DE3) (Novagen, Inc., Madison, WI, USA) using either the pPM7 vector [37], or the pET15b vector (Novagen, Inc., USA) encoding PAI-2 with a histidine-tag fused to the N-terminus (his-tagged PAI-2).

2.2. Purification of PAI-2 from E. coli

PAI-2 without his-tag was purified as described earlier [37]. For purification of the his-tagged PAI-2, the bacterial extract in 50 mM Tris–HCl, pH 7.0, 0.3 M NaCl was loaded on TALON beads (Clontech Laboratories, Inc., Palo Alto, CA, USA) equilibrated with the same buffer. Following washing with 50 mM Tris–HCl, pH 6.8, 0.3 M NaCl, 20 mM imidazole, PAI-2 was eluted with 50 mM Tris–HCl, pH 5.6, 200 mM imidazole, and stored with 25% glycerol at −20°C. The purified his-tagged wt PAI-2 inhibited uPA as efficiently as wt PAI-2 (data not shown).

2.3. Bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid) binding

The binding of bis-ANS to hydrophobic cavities was monitored by an increase of its fluorescence intensity. The fluorescence measurements were performed at 37°C using a FluoroMax-2 spectrofluorimeter (Instruments S.A., Inc., Edison, NJ, USA). Before the experiments, PAI-2 was desalted on a NAP-25 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with 50 mM sodium phosphate buffer, pH 5.9, 10% glycerol. An aliquot of 150 μl 1 M Tris–HCl, pH 8.0, was mixed with 10 μl bis-ANS (Molecular Probes, Eugene, OR, USA) (5 mM stock solution in dimethyl sulfoxide) and 900 μl of PAI-2 (70 μg/ml) and fluorescence changes were immediately measured at 520 nm upon exciting at 370 nm.

2.4. Intrinsic tryptophan fluorescence measurements

Purified PAI-2 was desalted on a NAP-25 column equilibrated with 50 mM sodium-phosphate buffer, pH 7.4, 0.14 M NaCl (PBS). The fluorescence changes of PAI-2 (60 μg/ml) were measured at 45°C by excitation at 295 nm and detection at 340 nm. Light scattering was measured under the same conditions at excitation and emission wavelengths of 400 and 405 nm, respectively.

2.5. General methods

The inhibitory activity of PAI-2 was determined by a chromogenic assay as previously described [37]. Both sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 10%) and native PAGE (10%) were performed as described [39], except that the native PAGE was run 5 h in SDS-free buffers. Following electrophoresis, the proteins were immuno-blotted [40] and PAI-2 antigen was detected with monoclonal antibodies MAI-21 (Biopool, Umeå, Sweden). Anti-mouse IgG conjugated to horseradish peroxidase (Promega, Madison, WI, USA) was used for detection by the enhanced chemiluminescence (ECL) method (Amersham Pharmacia Biotech AB, UK). PAI-2 polymerization was quantified from a native PAGE using a phosphoimager (GS-250 Molecular Imager™, Bio-Rad, Hercules, CA, USA). Protein concentrations were determined by the bicinchoninic acid assay (Pierce,Rockford, IL, USA).

3. Results and discussion

3.1. Expression and purification of PAI-2

When PAI-2 was purified according to our old protocol [37], as much as 47–60% of the purified protein was in the

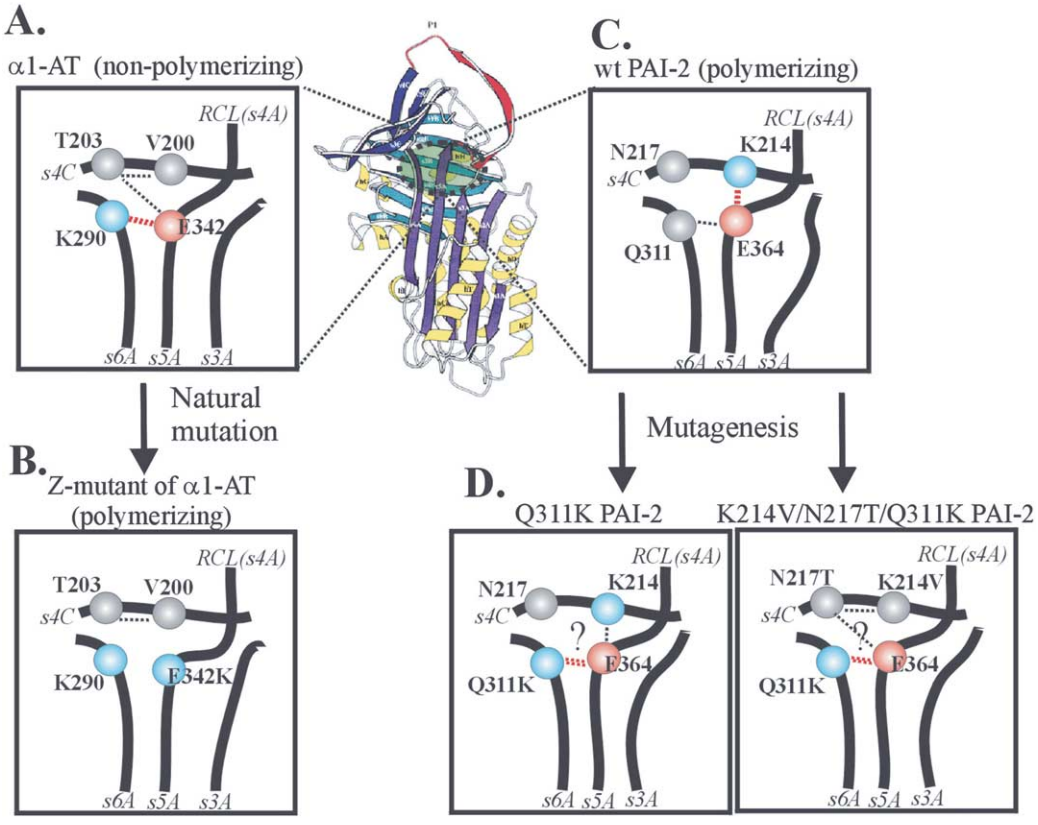

Fig. 1. A scheme of amino acid interactions in the breach region of α_1-AT and PAI-2. The ribbon model shows α_1-AT (adopted from [45]) with RCL in red, A β-sheet in violet, and the breach region as a green ellipse. Panels A–D show a closer view of the breach region. Salt bridges are marked as double-dotted red lines, and hydrogen bonds as dotted black lines. Lysine and glutamic acid residues are presented as blue and red balls, respectively. A: α_1-AT with the crucial salt bridge between K290 and E342. B: Z-form of α_1-AT with the E342K substitution. C: Wt PAI-2 with hydrogen bond between Q311 and E364, a salt bond between E364 and K214, and a broad gap between strains s3A and s5A. D: Single and triple mutants of PAI-2, where amino acid residues that are conserved in others serpins have been introduced.

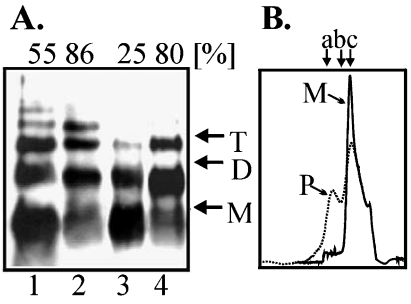

Fig. 2. Polymerization of wt PAI-2 with and without his-tag. A: Non-denaturing PAGE followed by Western blot: lane 1, non-modified wt PAI-2 purified according [37]; lane 2, non-modified wt PAI-2 incubated in PBS, pH 7.4, at 37°C for 18 h; lane 3, his-tagged wt PAI-2 purified by one-step affinity chromatography; lane 4, his-tagged wt PAI-2 incubated in PBS, pH 7.4, at 37°C for 18 h. The numbers above the lanes indicate the extent of polymerization (%). M, Monomers; D, dimers; T, trimers. B: Molecular sieving of his-tagged wt PAI-2, performed on SEC-400 column (Bio-Rad Laboratories AB, Hercules, CA, USA) equilibrated with PBS, pH 5.6, before (continuous line) and after (dotted line) the incubation at 37°C for 18 h. M, Monomers; P, polymers, arrows at the top indicate the migration of (a) dimer of bovine serum albumin, MW 136 kDa, (b) monomer of bovine serum albumin, MW 68 kDa, (c) monomer of ovalbumin, MW 43 kDa.

polymerized form when analyzed by native PAGE (Fig. 2A, lane 1). However, to study PAI-2 polymerization, purified preparations where the protein is mainly in the monomeric form are required. We therefore attached a his-tag to the N-terminus of PAI-2 to allow purification by a one-step procedure. After purification by this new protocol, approximately 5–27% of the his-tagged inhibitor was polymerized (Fig. 2A, lane 3). Both non-modified wt and his-tagged wt PAI-2 polymerized to about 80% following incubation at 37°C for 18 h, (Fig. 2A, lanes 2 and 4, respectively). In both the cases, the dominating forms were dimers and trimers, with a small amount of higher polymers detected. In this respect PAI-2 differs from antitrypsin, which requires partial denaturation to polymerize and then produces similar amounts of dimers, trimers and higher polymers [20]. Polymerization of PAI-2 was also analyzed by molecular sieving chromatography. As shown in Fig. 2B, purified his-tagged PAI-2 eluted from the column as a single peak corresponding to monomers and only trace amount of polymers (about 5%) was detected. After incubation at 37°C, a larger PAI-2 form corresponding to polymers (about 35% of total protein) appeared. The extent of polymerization was higher when the samples were analyzed by native PAGE, than by molecular sieving chromatography. This is most likely due to differences in pH during the analyses (will be discussed in Section 3.2). Taken together, our data indicate that the his-tagged PAI-2 has similar inhibitory activity and ability to polymerize as the non-modified wt protein, and therefore can be used to study polymerization in vitro.

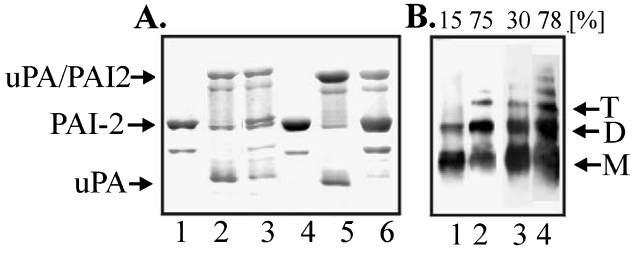

Fig. 3. Characterization of PAI-2 mutants. A: Complex formation between the mutants and uPA analyzed by reducing SDS–PAGE and Coomassie brilliant blue staining. Lane 1, purified Q311K mutant; lane 2, Q311K mutant incubated with 1.5 molar excess of uPA; lane 3, Q311K mutant pre-incubated for 18 h at 37°C before adding the uPA; lane 4, purified triple mutant (K214V/N217T/Q311K); lane 5, triple mutant incubated with 1.5 molar excess of uPA; lane 6, the triple mutant pre-incubated for 18 h at 37°C before adding the uPA. B: Polymerization of mutants analyzed by native PAGE followed by Western blot. The numbers above the lanes indicate extent of polymerization (%). Lane 1, purified Q311K mutant; lane 2, Q311K mutant incubated in PBS, pH 7.4, at 37°C for 18 h; lane 3, purified triple mutant; lane 4, the triple mutant incubated in PBS, pH 7.4, at 37°C for 18 h. M, Monomers; D, dimers; T, trimers.

3.2. Studies of breach-region mutants of PAI-2

The X-ray structures of α_1AT and other serpins reveal that the top part of the A β-sheet is stabilized by conserved amino acid interactions in the breach region [4,15–17,19,41]. In α_1AT, these interactions involve a salt bridge between K290 and E342, and a hydrogen bond between E342 and T203 (Fig. 1A). The three amino acids involved occupy homologous positions in more than 95% of the serpins from different species. However, PAI-2 is an exception since it has only one of the three residues, the glutamic acid (E364 in PAI-2), preserved [42] (Fig. 1C). As a consequence of this, PAI-2 has a hydrogen bond between residues E364 and Q311 that replaces the conserved salt bridge between the corresponding residues K290 and E342 in α_1AT, and a salt bridge between E364 and K214 that is not present in other serpins. The molecular deviations in the breach region may be the reason as to why the top part of the A β-sheet in PAI-2 has a broad gap between strands s3A (β-strand 3 of A β-sheet) and s5A, at the place of RCL insertion [42]. Since opening of the A β-sheet is an obligatory step for the polymerization process, it seemed reasonable that the absence of conserved amino acid interactions in the breach region of PAI-2 could be a cause for PAI-2 polymerization. To test this hypothesis, two his-tagged PAI-2 mutants were constructed. In one mutant (Q311K), the ability to form a salt bridge with E364 was established (Fig. 1D, left panel), and in the other mutant (K214V/N217T/Q311K, defined here as tri-

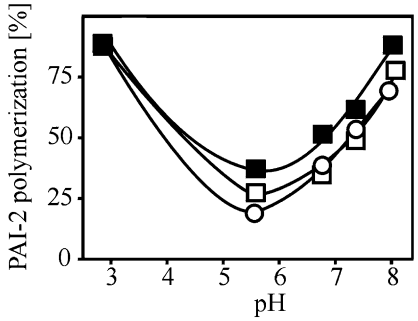

Fig. 4. The pH-dependence of PAI-2 polymerization. Wt PAI-2 and mutants were desalted on NAP-25 columns equilibrated with different buffers (0.1 M glycine, pH 2.9; 50 mM sodium phosphate, pH 5.6; 50 mM Tris–HCl, pH 6.8; 50 mM sodium phosphate, pH 7.4; 50 mM Tris–HCl, pH 8.0), all containing 0.14 M NaCl. Immediately after the filtration, the samples (85 μg/ml) were assayed by native PAGE followed by ECL-detection and quantification of the polymerization by using phosphoimager: □ - wt PAI-2; ○ - Q311K mutant of PAI-2; and ■ - triple PAI-2 mutant.

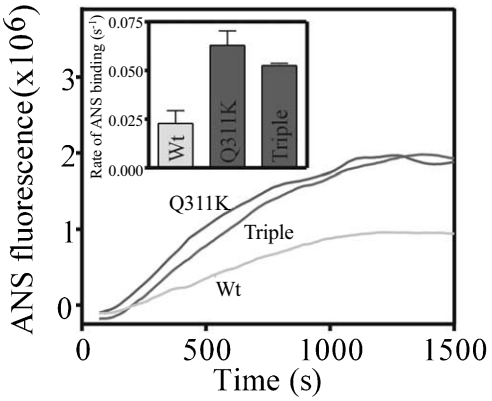

Fig. 5. Kinetics of bis-ANS binding to the wt and mutant forms of PAI-2. Monomeric PAI-2 proteins were mixed with bis-ANS and the fluorescence was measured by exciting at 370 nm and measuring emitted light at 520 nm. Insert, calculated rate of bis-ANS binding to PAI-2 (mean value of three experiments).

ple mutant) the molecular architecture found in most non-polymerizing serpins, including α_1AT, was established (Fig. 1D, right panel). The purified mutant proteins (Fig. 3A, lanes 1 and 4) could form complexes with uPA (Fig. 3A, lanes 2 and 5), indicating that the amino acid substitutions did not influence the inhibitory activity of PAI-2. Also, similar to wt protein, the mutants lost their ability to form complex with uPA after incubation at 37°C (Fig. 3A, lanes 3 and 6). When analyzed by non-denaturing PAGE, the mutant proteins were only slightly pre-polymerized after the purification (Fig. 3B, lanes 1 and 3), but polymerized to about 70–80% upon incubation at 37°C (Fig. 3B, lanes 2 and 4). The only difference between the mutants was that the Q311K mutant always polymerized to a lesser extent with the dimer as dominating oligomeric species, while the triple mutant revealed more higher polymers. Taken together, our data suggest that PAI-2 mutants with a molecular architecture found in the breach region of most non-polymerizing serpins, are still prone to polymerize.

To further investigate the mechanism of PAI-2 polymerization and the role of the breach region, we determined the pH-dependence of the polymerization reaction. As shown in Fig. 4, polymerization of wt PAI-2 and the two mutants was pH-dependent and revealed a similar bell-shaped pattern, with the lowest degree of polymerization at pH 5.6. Kinetics of the polymerization was similar for all the PAI-2 forms with highest rate at pH values 2.9, 7.4 and 8.0, and with most of the protein polymerizing within 1 h (data not shown). When analyzed by native PAGE, the proteins revealed a distribution of dimers, trimers and higher polymers analogous to that shown in Fig. 2, lane 4 for wt PAI-2 and in Fig. 3B, lanes 2 and 4 for Q311K and the triple mutant (respectively). A similar pH-dependent polymerization reveals the wt form of α_1-AT [43]. However, in contrast to PAI-2 that spontaneously polymerizes under physiological conditions, α_1-AT polymerizes only when the protein is destabilized by the extremes of pH. Since a decrease in pH from neutral to slightly acidic is accompanied by protonation of imidazole groups, it is possible that histidine(s) in PAI-2 might be involved in the stabilization of the monomeric form seen at pH 5.6. A potential candidate could be the histidine at position P3′ of the RCL, which might be involved in a pH-dependent regulation of the polymerization process. Similarly, the conversion of PAI-1 from the active to the latent form is a pH-dependent process, where histidine residues are implicated in the stabilization of the active PAI-1 at pH 5.6 [44].

3.3. Studies of conformational changes in PAI-2 mutants by bis-ANS binding

Hydrophobic cavities have been identified on the surface of several serpins and the volumes of these cavities have been shown to correlate with the opening of the A β-sheet which takes place during the conversion of active serpins to the latent or cleaved forms [45,46]. To study if mutations in the breach region of PAI-2 induce changes in hydrophobic cav-

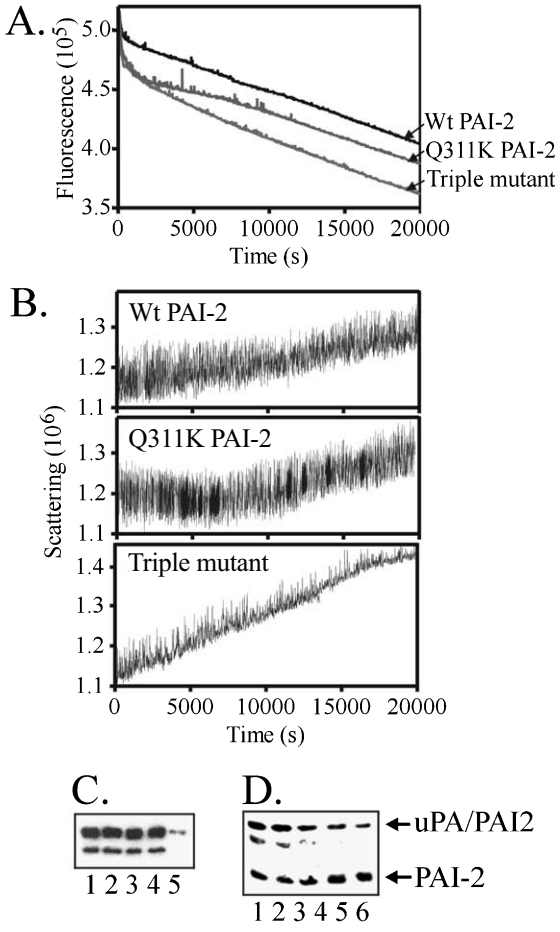

Fig. 6. Polymerization of wt and mutant forms of PAI-2 at 45°C. A: Kinetics of intrinsic tryptophan fluorescence of wt PAI-2, the Q311K mutant and the triple mutant in PBS, pH 7.4, obtained by exciting the fluorophore at 295 nm and measuring emitted light at 340 nm (mean of two to three experiments). B: Light scattering of the same PAI-2 samples measured with emission wavelength at 400 nm and detection wavelength at 405 nm. C: The heat-stability of wt PAI-2. At different times of incubation at 45°C, PAI-2 samples were centrifuged for 1 h, filtered through a 0.22 μm filter, and analyzed (30 μl samples) on SDS–PAGE followed by Western blot. Lane 1, PAI-2 before the incubation; lanes 2, 3, 4, 5, PAI-2 after 1, 3.5, 5.5, 18 h of incubation (respectively). D: Inhibitory activity of wt PAI-2 determined at different times of incubation at 45°C by complex formation with 1.5 molar excess of uPA. Lane 1, PAI-2 before the incubation; lanes 2, 3. 4, 5, 6, PAI-2 after 1, 2, 3, 4, 5.5 h of incubation (respectively).

ities, we performed binding studies using the dye bis-ANS. As shown in Fig. 5, the initial rate of bis-ANS binding, as well as the amount of the dye bound, were much higher for the Q311K and the triple mutants of PAI-2, as compared to wt PAI-2. Light scattering of the PAI-2 samples, measured in parallel to the bis-ANS binding, did not change during the time of the experiments, and the protein remained inhibitory active (data not shown), indicating that the measured fluorescence of bis-ANS resulted from interaction of the probe with monomeric forms of PAI-2. Based on the correlation between the amount of bis-ANS bound and structural rearrangements in serpins [46], our data indicate that the breach-region mutations cause conformational changes in PAI-2, and possibly influence the opening/closing of the A β-sheet.

3.4. Kinetics of PAI-2 polymerization

To determine the kinetics of PAI-2 polymerization, changes of intrinsic tryptophan fluorescence and light scattering were measured over time at 45°C. As shown in Fig. 6A, the fluorescence of wt PAI-2 decreased linearly with a rate of 4.47 ± 0.32 s^{-1} and this decay was accompanied by a linear increase of light scattering (Fig. 6B), suggesting that the observed changes in fluorescence are linked to PAI-2 polymerization. The overall pattern of the triple mutant with linear changes of fluorescence decay and light scattering was similar to wt PAI-2. However, the rate of fluorescence decay (5.7 ± 0.51 s^{-1}) and the increase in light scattering were faster, suggesting that the three mutations destabilize the PAI-2 molecule and make it even more prone to polymerize than wt PAI-2. The linearity of the changes implies that wt PAI-2 and the triple mutant do not require any conformational rearrangements before polymerization can take place. The Q311K mutant revealed a different pattern. With this mutant, the fluorescence decay had an initial slower phase (with a rate of 2.52 ± 0.39 s^{-1}), during which no changes of light scattering were observed, suggesting that this phase corresponds to unimolecular conformational rearrangements. However, the subsequent faster phase of fluorescence decay (rate 4.78 ± 0.51 s^{-1}) was accompanied by a linear increase of the light scattering, indicating that the polymerization takes place during this phase. The data therefore suggest that the Q311K mutant requires conformational transitions before polymerization can be initiated, after which the mutant polymerizes like the wt inhibitor. To exclude the possibility of protein precipitation during the experiment, we compared the concentration of PAI-2 before and following different times of incubation at 45°C. As it is shown for the wt PAI-2 (Fig. 6C), the PAI-2 concentration remained stable for at least 5.5 h (i.e. the time of the experiment), and precipitation was only seen after prolonged (18 h) incubation. The Q311K and the triple mutants had similar thermal stability as the wt protein (data not shown). In parallel with the fluorescence measurements, we determined PAI-2 activity by complex formation with uPA. As shown in Fig. 6D for wt PAI-2, the amount of the complex decreased and amount of inactive PAI-2 increased during the incubation, indicating that the decay of fluorescence was due to formation of polymers. A loss of the inhibitory activity, which accompanied the decay of tryptophane fluorescence, was also observed for the PAI-2 mutants (data not shown). A similar two-phased polymerization process has been described for α_1AT [43]. However, in contrast to the small differences between wt PAI-2 and its mutants observed here, the rate of polymer formation by the Z-α_1AT mutant was 19-fold faster than this for the wt protein.

In summary, our study reveals that wt PAI-2 exists in a highly polymerogenic conformation and does not require structural rearrangements before polymerization can take place. Since an opening of the A β-sheet is obligatory for serpins to polymerize, we propose that wt PAI-2 has a more open A β-sheet than other serpins. Unexpectedly, substitutions in the breach region designed to stabilize the monomeric form of PAI-2 resulted in a conformation with retained ability to polymerize. Our data therefore suggest that deviations that disrupt conserved amino acid interactions in the breach region (Fig. 1C) are not the molecular cause for the spontaneous polymerization of PAI-2.

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