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Type-1 inhibitor of plasminogen activators

Distinction between latent, activated and reactive centre-cleaved forms with thermal stability and monoclonal antibodies

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Type-1 inhibitor of plasminogen activators (PAI-1) occurs in purified preparations in a latent form that can be activated with denaturants; in vivo, latency is prevented by binding to vitronectin. We have compared latent, denaturant-activated and reactive centre-cleaved human PAI-1 with respect to thermal stability and affinity to monoclonal antibodies. By both criteria, latent and cleaved PAI-1 are very similar or indistinguishable, and clearly different from active PAI-1. Our findings suggest that the conformations of latent and reactive centre-cleaved PAI-1 are similar and resemble the so-called relaxed (*R*) serpin conformation, while that of active PAI-1 is different and resembles the stressed (*S*) serpin conformation.

Serpin; Plasminogen activator; PAI-1; Serine proteinase

1. INTRODUCTION

The serpins constitute a superfamily of extracellular glycoproteins, many of which are inhibitors of serine proteinases. They expose at their surface a 20 amino-acid-long peptide segment, situated near the C-terminus. In the inhibitory serpins, this segment contains a peptide bond called the reactive centre, between amino acids denoted P_1 and P_1'. Proteinase interaction with the reactive centre leads to the formation of a 1:1 stoichiometric complex. Treatment of the complexes with nucleophilic agents under denaturing conditions results in the dissociation and release of reactive centre-cleaved, inactive inhibitors. In non-inhibitory serpins, for instance ovalbumin, the exposed reactive centre segment analogue is particularly susceptible to proteolytic cleavage [1].

The X-ray crystal structures of reactive centre-cleaved α_1-proteinase inhibitor (α_1-PI) [2], native ovalbumin [3], elastase-cleaved ovalbumin (plakalbumin) [4] and reactive centre-cleaved α_1-antichymotrypsin [5] have been determined. Based on sequence alignment of different serpins [1], these three-dimensional structures may be used for discussion of the three-dimensional structures of serpins in general. In native ovalbumin, the reactive centre segment is exposed and separated from the main body of the molecule; N-terminally, it is connected to strand 5 of a large central β-sheet termed A. In cleaved α_1-PI and α_1-antichymotrypsin, the peptide segment P_1–P_{15} is inserted as strand 4 of β-sheet A. In contrast, in cleaved ovalbumin, the liberated segment remains exposed (see Fig. 1).

Reactive centre-cleavage of inhibitory serpins is associated with a marked increase in thermal stability (in the sense of an increase in solubility at elevated temperatures) and changes in spectroscopic properties [6–9]. The conformational forms before and after cleavage have been termed stressed (*S*) and relaxed (*R*), respectively. In contrast, cleavage of ovalbumin does not lead to such changes [7,10,11]. Thus, conversion of the *S* to the *R* conformation appears to be closely coupled to the presence of the segment P_1–P_{15} as strand 4 of β-sheet A.

Type-1 inhibitor of plasminogen activators (PAI-1) is a member of the serpin superfamily [12,13]. When purified, it appears in a latent form with a very low specific inhibitory activity. Latent PAI-1 can be activated by exposure to denaturants [14,15]. The active state of PAI-1 can be maintained by binding to vitronectin [16–20]. The molecular mechanism behind the latency of PAI-1 is unknown. In the present communication, we attempt to relate the latency of PAI-1 to the notion of *S* and *R* serpin conformations.

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2. MATERIALS AND METHODS

2.1. *Proteins and antibodies*

Human PAI-1 was purified from serum-free conditioned medium of dexamethasone-treated HT-1080 cells by immunoaffinity chroma-

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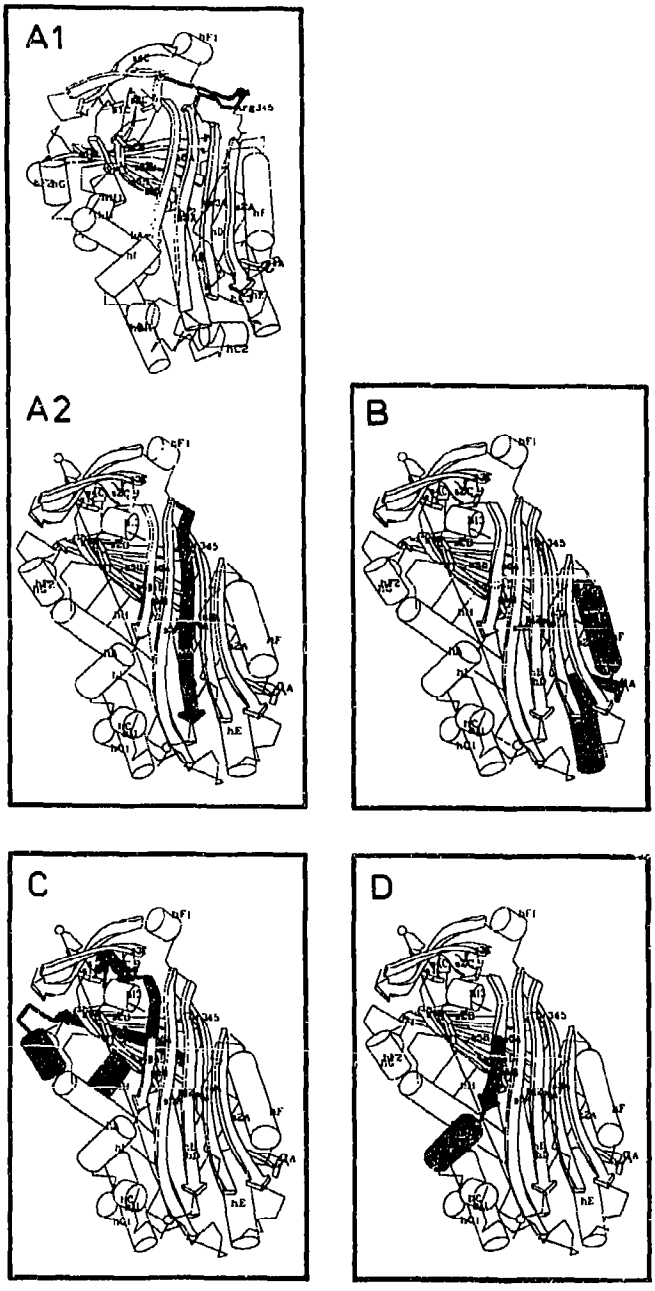

Fig. 1. Stereoscopic drawings of secondary structural elements of plakalbumin and cleaved α_1-PI. A1, plakalbumin; A2, B, C and D, reactive centre-cleaved α_1-PI. The peptide segment spanning the 15 amino acids N-terminal to the reactive center (P_1–P_{15}) is shaded in A. Note that 6 amino acids (P_1–P_6) have been cleaved from plakalbumin by the elastase treatment. The secondary structural elements indicated in B, C and D are those expected to contain the epitopes for monoclonal anti-PAI-1 antibodies from hybridoma clone 2 (B); from clone 3 (C); and from clone 5 (D), respectively, under the assumption that α_1-PI and PAI-1 contain the same secondary structural elements. h = α-helix, s = strand in a β-sheet. The stereoscopic drawings are adapted from Wright et al. [4], with permission. For further details about the epitopes, see Table I.

tography on a column of Sepharose 4B coupled with monoclonal murine anti-human PAI-1 IgG from hybridoma clone 2 [21]. Immediately after purification, the preparation was dialysed against 10 mM Na_2HPO_4, pH 7.4, 0.15 M NaCl (phosphate-buffered saline, PBS) and concentrated to 500–1200 μg/ml by the use of a Sartorius collodion bag at 4°C. It was stored in aliquots at −80°C. Thawed aliquots were not refrozen but discarded.

PAI-1 was activated with guanidinium hydrochloride by mixing stock solutions with an equal volume of 8 M guanidinium hydrochloride and incubating the mixture at room temperature for 2 h. The guanidinium hydrochloride was removed by an overnight dialysis against PBS.

PAI-1 was activated with SDS by incubating stock solutions with 0.1% SDS for 1 h at room temperature; the SDS was then quenched by the addition of Triton X-100 to a concentration of 1% and a more than 10-fold dilution with buffer with 0.1% Triton X-100.

Reactive centre-cleavage was performed by incubating undiluted SDS-activated PAI-1 (that is, containing 0.1% SDS, 1% Triton X-100) with human μ-PA (Serono, Switzerland) at a concentration of 0.5 μg/ml for 4 h at 37°C [22,23]. This was followed by a more than 10-fold dilution with buffer with 0.1% Triton X-100. In some cases, PAI-1 was cleaved before purification, directly in the conditioned medium. SDS-activation and μ-PA treatment (as above) were followed by purification as above, only with an extra wash with 0.1 M Tris, pH 8.1, 1% Triton X-100, in order to ensure complete removal of SDS.

3 clones of hybridomas producing anti-human PAI-1 IgG, termed clones 1–3, were those described previously [21]. Hybridoma clone 5 resulted from an independent immunization and fusion [Nielsen, L.S. and Andreasen, P.A., unpublished]. Polyclonal rabbit anti-human PAI-1 IgG was as before [24].

2.2. *Measurement of thermal stability*

The different forms of PAI-1 were incubated at the indicated temperatures for 2 h at a concentration of 75 μg/ml. After incubation, the samples were cooled on ice and centrifuged at 20 000 × g. Aliquots corresponding to 10 μg PAI-1 were taken from the supernatants and subjected to SDS-PAGE in gels with 10% polyacrylamide. Coomassie blue-stained gels were scanned spectrophotometrically with a Shimadzu Dual Wavelength Scanner CS930.

2.3. *Enzyme linked immunosorbent assays (ELISA)*

To test the binding of different PAI-1 forms to monoclonal antibodies, the various monoclonal antibodies were coated onto the solid phase. A layer of PAI-1 in various concentrations was followed by a layer of polyclonal rabbit anti-PAI-1 IgG and a layer of peroxidase-conjugated swine IgG against rabbit IgG. To test for differences in epitopes between monoclonal anti-PAI-1 IgG from different hybridoma clones, the solid-phase antibody was monoclonal IgG from one of the various hybridoma clones, as above. A layer of PAI-1 in various concentrations was followed by a layer of biotin-labelled monoclonal IgG from one of the various hybridoma clones and a layer of avidin-peroxidase. The members of pairs of antibodies showing compatibility of binding were scored as having different epitopes. Other details of the ELISAs were as described earlier [25].

3. RESULTS

3.1. *Thermal stability*

We characterized latent, activated and reactive centre-cleaved PAI-1 by thermal stability in the sense of solubility at a given temperature. Cleaved PAI-1 remained in solution at temperatures up to 90°C (Fig. 2C). In contrast, guanidinium hydrochloride- and SDS-activated PAI-1 was labile at temperatures above 40°C (Fig. 2B). Latent PAI-1 was, when incubated at temperatures above 40°C, partially converted to 3 types of aggregates migrating in SDS-PAGE corresponding to M_r of approximately 87 000, 107 000 and 120 000, respectively. However, the total amount of protein seen

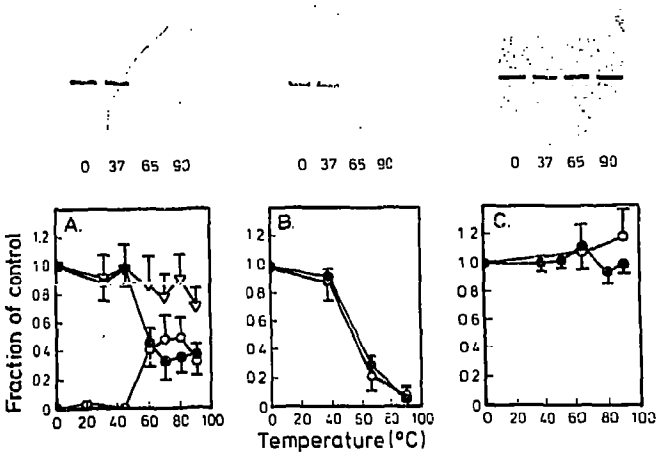

Fig. 2. Thermal stability of different forms of PAI-1. The different forms of PAI-1 was incubated at various temperatures for 2 h. If not otherwise indicated, the buffer was 0.1 M Tris-HCl pH 8.1, 0.1% Triton X-100. After incubation, cooling on ice and centrifugation, the amount of PAI-1 remaining in solution was analysed by SDS-PAGE and Coomassie blue staining (top part of the figure). The gels were scanned spectrophotometrically (bottom part of the figure). For each PAI-1 form, the amounts found on the gels after the incubations were expressed as a percentage of that found in a sample kept at 0°C (bottom part of figure). A: Latent PAI-1. (●), M_r 54 000 band; (○), higher M_r bands; (▽), sum of all bands. B: Activated PAI-1. (○), guanidinium hydrochloride-activated PAI-1; (⊜), SDS-activated PAI-1 (final SDS-concentration during the incubations 0.0062%, final Triton X-100 concentration 0.168%). The gel shows guanidinium hydrochloride-activated PAI-1. C: Cleaved PAI-1. (●), PAI-1 cleaved after purfication (final SDS concentration during the incubations 0.010%, final Triton X-100 concentration 0.191%); (○), PAI-1 cleaved in the conditioned medium before purification; there was no SDS present during the incubations. Mean and standard deviation are indicated. The number of determinations were between 2 and 4. The SDS and the additional Triton X-100 in SDS-activated PAI-1 and one of the preparations of cleaved PAI-1 were carried over from the SDS-activation. The identity of the results obtained with 1, PAI-1 cleaved before and after purification, and 2, PAI-1 activated with SDS and guanidinium hydrochloride, demonstrate that these small amounts of SDS did not influence the results obtained. Separate control experiments (not shown) demonstrated that SDS in these low concentrations also did not affect the thermal stability of latent PAI-1.

on the gels remained almost constant at all temperatures (Fig. 2A). Also, latent PAI-1 migrated in gel filtration on a Superose 12HR-column (PBS, room temperature) as a single, sharp peak to the same position as ovalbumin (not shown), indicating the absence of aggregation at lower temperatures. Activated PAI-1 migrated into the same position.

3.2. *Reactivity with monoclonal antibodies*

We used 4 monoclonal anti-human PAI-1 antibodies. The epitopes for 3 of the antibodies have been localized to certain stretches of the polypeptide chain [26]. By the use of the three-dimensional structure of cleaved α_1-PI [2], and the sequence alignment of different serpins given by Huber and Carrell [1], these epitopes may be assigned to different secondary structural elements which, due to the strong sequence homology, are expected to exist in both PAI-1 and α_1-PI (Table I). Antibodies from clone 2, and from clones 3 and 5, respectively, are seen to bind to epitopes localized on opposite faces of the molecule (Fig. 1). The epitope for antibodies from clone 1 could not be assigned to a distinct, linear area of PAI-1. We also found, by ELISA, that any pair of the 4 antibodies was able to bind at the same time (data not shown), and thus all antibodies have different epitopes.

Table I

Properties of murine monoclonal antibodies against human PAI-1

Hybridoma clone, no.	Epitope, amino acids	Epitope, secondary structural elements
1	not linear	
2	110—145	hE, s1A, hF
3	235—283	s3B, hG, hH, s2C, s6A
5	284—295	s6A, hI

'h' denotes an α-helix and 's' a strand in a β-sheet. The numbering of the secondary structural elements is that given by Löbermann et al. [2]. Their locations are indicated in Fig. 1. See the text for further explanation.

The binding of latent, activated and reactive centre-cleaved PAI-1 to the monoclonal antibodies was tested by ELISA. IgG from clone 2 reacted identically with the different forms (not shown). With IgG from hybridoma clones 3 and 5, the binding to latent PAI-1 was weak, but increased strongly upon activation, while it decreased to the level of the latent form upon reactive centre-cleavage. With IgG from hybridoma clone 1, the affinity changed in the reverse manner (Fig. 3). Testing the binding over a wider PAI-1 concentration range (not shown), the difference was found to be 5-fold in the case of IgG from clone 1, and more than 100-fold with IgG from clones 3 and 5.

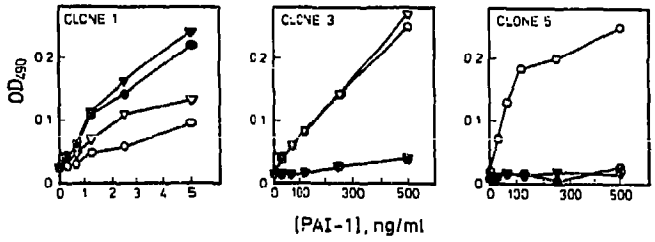

Fig. 3. Binding of different forms of PAI-1 to monoclonal IgG. The binding of latent (●), SDS-activated (○), guanidinium hydrochloride-activated (▽), and reactive centre-cleaved PAI-1 (▼) to anti-PAI-1 IgG from hybridoma clone 1, clone 3 and clone 5 was measured by ELISA. The concentrations of PAI-1 in the assay wells are indicated on the abscissa, and the resulting optical density at 490 nm at the ordinate. The buffer was PBS with 0.01% Tween 20. Small amounts of SDS (<0.0001%) were carried over from the stock solutions of SDS-activated and cleaved PAI-1. The possibility for an effect of this directly on binding was excluded by adding similar amount of SDS to latent and guanidinium hydrochloride activated PAI-1, the only difference being that the Triton X-100 used to quench the SDS was added before SDS in these cases.

4. DISCUSSION

Our finding of a high thermal stability of reactive centre-cleaved PAI-1 shows that this is in the *R*-conformation, and suggests that reactive centre-cleavage is associated with expansion of β-sheet A by a sixth strand, consisting of amino acids P_1–P_{15}, also in this serpin. In contrast, activated PAI-1 appears to be in the *S* conformation like the native forms of other inhibitory serpins. The thermal stability of latent PAI-1 suggests that this form is also in an *R*-like conformation. The similarity between latent and cleaved PAI-1 is substantiated by their reactivity with monoclonal antibodies, by which criterion they are indistinguishable and clearly different from activated PAI-1. On the basis of this similarity, one may speculate that the peptide segment P_1–P_{15} is partially inserted into β-sheet A in latent PAI-1. As pointed out by Stein et al. [3], a partial insertion is possible, due to the fact that the reactive centre loop is partly in the form of an extensible α-helix. A partial insertion could result in the reactive centre segment becoming inaccessible to the proteinase by placing the uninserted part of it very close to the main body of the molecule. This proposal is in agreement with recent findings of Schulze et al. [27] showing that a peptide corresponding to P_1–P_{15} of α_1-PI can convert native α_1-PI to a form resembling the reactive centre-cleaved form by several physicochemical parameters.

The tendency of latent PAI-1 to form aggregates at high temperatures suggests that differences do exist between the conformations of latent and cleaved PAI-1, since the latter does not form aggregates. However, there were no indications of aggregation at lower temperatures, neither by SDS-PAGE nor gel filtration, and latent PAI-1 migrated in the same position as activated PAI-1 in gel filtration. This shows that the observed properties of latent PAI-1 is not determined by its tendency to form aggregates.

The epitopes for anti-PAI-1 antibodies from clones 3 and 5, which are most exposed in the activated form, are seen to be present in regions of the molecule that may possibly be affected by changes concerning β-sheet A. The region contains strand 6A, a member of this β-sheet (see Fig. 1). In addition, the region contains strand 2C with Lys^{279} (Lys^{290} in α_1-PI), which appears to form a salt bridge to Glu^{332} (Glu^{342} in α_1-PI); Glu^{332} is at the pivot point at the insertion of strand 4A into β-sheet A [1]. Antibodies from clone 2 are not conformation-sensitive, although their epitope is localized to a region in proximity to β-sheet A. The epitope for antibodies from clone 1, which is most exposed in the latent and cleaved forms, is probably formed by amino acids from different parts of the polypeptide chain, but it does not overlap with those of antibodies from the three other clones.

The epitopes tested in the present work represents most of the epitopes found for monoclonal antibodies from a number of laboratories [26]. It may be of interest to test if antibodies with other epitopes, produced for instance by immunization with peptides, are sensitive to other conformational changes.

In vivo, vitronectin appears to function as a cofactor for PAI-1, stabilizing it in an active conformation [17–21]. Extending the above proposal, the function of vitronectin would be to prevent the partial insertion of the intact reactive centre segment into the central β-sheet, thereby keeping it accessible to the proteinase.

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