The vitronectin binding area of plasminogen activator inhibitor-1, mapped by mutagenesis and protection against an inactivating organochemical ligand

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Abstract A distinguishing feature of serpins is their ability to undergo a conformational change consisting in insertion of the reactive centre loop (RCL) into β-sheet A. In the serpin plasminogen activator inhibitor-1 (PAI-1), RCL movements are regulated by vitronectin, having a previously poorly defined binding site lateral to PAI-1's β-sheet A. Using a novel strategy, based on identification of amino acid residues necessary for vitronectin protection of PAI-1 against inactivation by 4,4′-dianilino-1,1′-bisnaphthyl-5,5′-disulfonic acid, we have defined a vitronectin binding surface spanning 10 residues between α-helix F, β-strand 2A, and α-helix E. Our results contribute to elucidating the unique serpin conformational change. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Bis-ANS; Plasminogen activator inhibitor-1; Plasminogen; Serpin; Vitronectin

1. Introduction

The serpins constitute a protein family of which the best characterised members, including plasminogen activator inhibitor-1 (PAI-1), are inhibitors of mammalian serine proteinases. Decisive for the inhibitory mechanism of serpins is the surface-exposed, approximately 20 amino acids long reactive centre loop (the RCL) (see Fig. 1). The reaction between serpin and proteinase is initiated by formation of a docking complex in which the P_1–P_1' bond in the RCL interacts non-covalently with the active site of the proteinase. In the ensuing locking step, the P_1–P_1' bond is cleaved, the P_1 residue coupled to the active site serine of the proteinase by an ester bond, the N-terminal part of the RCL inserted as strand 4 in β-sheet A (s4A), and the proteinase translocated to the opposite pole of the molecule. The proteinase is thereby distorted and the completion of the catalytic cycle halted. The energy needed for proteinase distortion stems from stabilisation of the serpin in the 'relaxed' conformation by insertion of the RCL into β-sheet A, as compared to the 'stressed', unstable active conformation with a surface-exposed RCL. At some conditions, proteinase distortion cannot keep pace with ester bond hydrolysis, resulting in abortive complex formation, full cleavage of the P_1–P_1' bond, insertion of the RCL into β-sheet A and release of an active proteinase. Serpins following this alternative path are said to exhibit substrate behaviour (for reviews, see [1–3]).

RCL insertion is coupled to conformational changes in other parts of the molecule, including the flexible joint region around α-helix D (hD) and hE [4] (see Fig. 1). A few organochemical compounds able to neutralise PAI-1 activity bind to the flexible joint region, including bis-ANS (4,4′-dianilino-1,1′-bisnaphthyl-5,5′-disulfonic acid), which inactivate PAI-1 by inducing substrate behaviour followed by polymerisation [5–6].

PAI-1 possesses two unique features not shared with other serpins. First, it spontaneous assumes an inactive, relaxed, so-called latent state in which the intact RCL is inserted into β-sheet A [8]. Second, stressed, but not relaxed PAI-1, binds the N-terminal 44 amino acids long somatomedin B domain of the M_r 70 000 protein vitronectin (VN), which thereby delays the latency transition of PAI-1 (for a review, see [3]) and protects it from bis-ANS-induced substrate behaviour and polymerisation [6–7]. VN causes a conformational change of the RCL [9–10]. VN binds to the flexible joint region of PAI-1, as suggested by four lines of evidence. By screening PAI-1 variants produced by random mutagenesis, the substitutions L118P and Q125K were found to result in a significantly reduced VN binding [11]. A recent report implicated residues 116–120 in VN binding and confirmed the reduced VN affinity of PAI-1 Q125K [12]. Investigations with the less accurate methods of antibody competition [13] and PAI-1-PAI-2 chimeras [14] also gave results compatible with VN binding to the flexible joint region. Thus, there seems to be a bidirectional communication between the VN binding area in the flexible joint region and the RCL.

However, the exact architecture of the VN binding site, i.e., the identity of the PAI-1 residues at the PAI-1–VN interphase, has remained elusive. We have now employed site-directed mutagenesis and the competitive effects of VN and bis-ANS for obtaining a map of the VN binding area.

2. Materials and methods

2.1. Materials

The cDNAs for wild-type (wt) and substituted human PAI-1, extended at the N-terminus with a hexa-His tag and a recognition motif

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Abbreviations: bis-ANS, 4,4′-dianilino-1,1′-bisnaphthyl-5,5′-disulfonic acid; h, α-helix; PAI-1, plasminogen activator inhibitor-1; s, β-strand; uPA, urokinase-type plasminogen activator; VN, vitronectin

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for heart muscle kinase, were produced by standard methods in the *Escherichia coli* expression vector pT7-PL [15]. Transformed *E. coli* BL21(DE3)pLysS cells from 1 l cultures, treated with 0.5 mM isopropyl thio-β-D-galactoside to induce PAI-1 expression, were harvested by centrifugation (7000×*g*, 20 min), re-suspended in 35 ml phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4), and disrupted by sonication. The homogenates were centrifuged (10 000×*g*, 30 min), filtered (0.22 μm), supplemented with 2 M NaCl, 10 mM imidazole and 5% glycerol, and applied to a 5 ml Ni-NTA column equilibrated in the same buffer. PAI-1 was eluted with 200 mM imidazole. The eluted protein was subjected to gel filtration on a Superdex 75 column (1.6×60 cm) equilibrated in HEPES-buffered saline (10 mM HEPES, 140 mM NaCl, pH 7.4) supplemented with 5% glycerol and 1 M NaCl. The procedure routinely gave 10–15 mg PAI-1/l bacterial culture. The preparations contained PAI-1 which was more than 95% pure as evaluated by SDS–PAGE and Coomassie blue staining. N-terminal sequencing showed the expected N-terminus, i.e., (M)GSMGSHHHHHHGSRRASV-HH..., only missing the initiating M, indicated in parentheses. The N-terminal extension did not affect PAI-1's specific inhibitory activity, its second order rate constant for reaction with urokinase-type plasminogen activator (uPA), its VN binding, or its rate of latency transition.

The PAI-1 amino acid numbering system used was that described earlier, based on the determination of the N-terminal amino acid sequence of the protein as S1-A2-V3-H4-H5... [16].

Bis-ANS was from Molecular Probes, Eugene, OR, USA. S-2444 (pyro-Glu-Gly-Arg-p-nitroanilide) was from Chromogenix, Mölndal, Sweden. Human uPA was from Wakamoto Pharmacautical Co., Tokyo, Japan. Human multimeric VN was from BD Biosciences, Franklin Lakes, NJ, USA.

2.2. Measurement of the specific inhibitory activity of PAI-1

To measure the effects of VN and bis-ANS on the specific inhibitory activity of wt and substituted PAI-1, i.e., the fraction of inhibitor forming a stable complex with uPA, PAI-1 was preincubated for 10 min on ice at concentrations of 10–30 μg/ml, in the absence or presence of a 1.5-fold molar excess of VN. It was then serially diluted at 37°C with HEPES-buffered saline supplemented with 0.25% gelatin, resulting in PAI-1 concentrations between 0.02 and 40 μg/ml in a volume of 100 μl. 50 μl aliquots of bis-ANS solution were added, the bis-ANS concentration varying between dilution series. The mixtures were incubated for 10 min. 50 μl aliquots with 1 μg/ml uPA were added. Incubation was continued for at least 2 min, sufficient for the process of inhibition of uPA to come to an end. The remaining uPA enzyme activity was determined by incubation with the substrate S-2444 and measurement of the increase in absorbance at 405 nm. The specific inhibitory activity of PAI-1 was calculated from the amount of PAI-1 that had to be added to inhibit 50% of the uPA. The IC_{50} values for bis-ANS neutralisation of PAI-1 were determined as the bis-ANS concentrations causing a 50% reduction in the PAI-1 specific inhibitory activity.

3. Results

Among about 40 A-substituted variants of PAI-1, produced to localise its VN-binding area, a substantial fraction had a reduced inhibitory activity, because of either an increased tendency to substrate behaviour or an increased fraction of PAI-1 being in an inert, probably latent state (data not shown). It was thus clear that the results of a direct VN–PAI-1 binding analysis would be misleading, as any relaxed form of PAI-1 does not bind to VN.

We therefore chose an alternative approach, intending to measure the interaction of VN with only the fraction of active

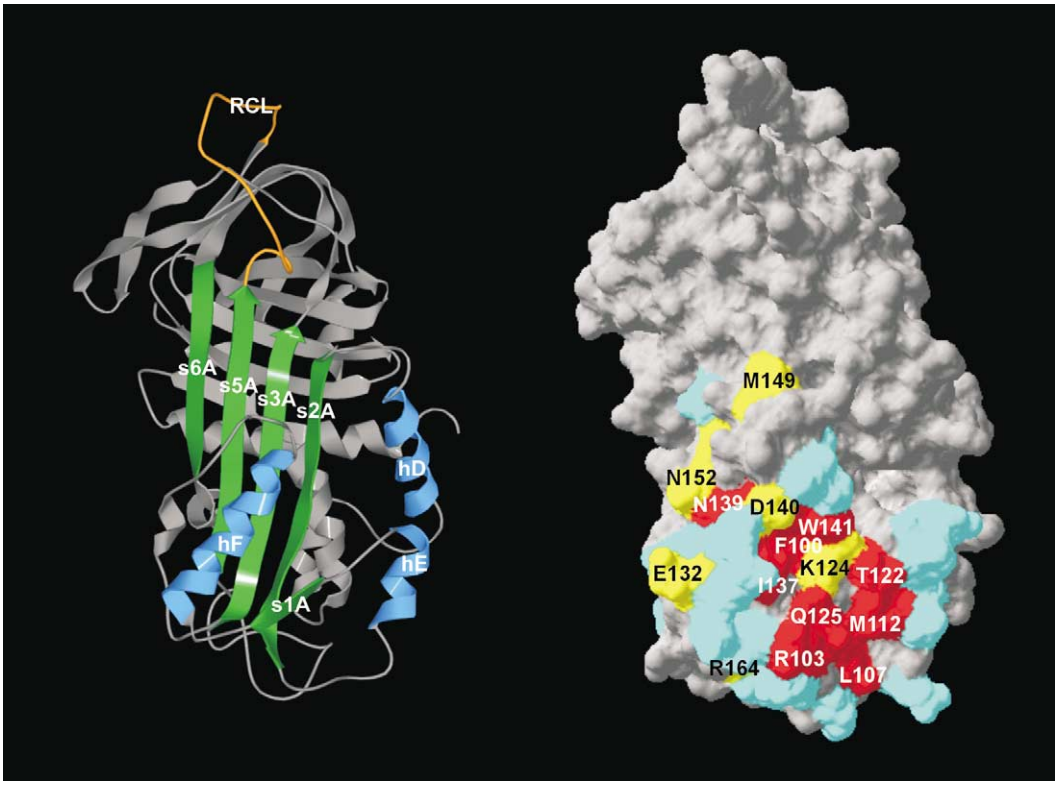

Fig. 1. The three-dimensional structure of active PAI-1 [20] and residues important for VN protection of PAI-1 against bis-ANS. A: Ribbon diagram. Some important secondary structural elements are indicated. B: Surface diagram, in the same orientation as in A. Substitution of the red residues resulted in an IC_{50} for bis-ANS inactivation of PAI-1 in the presence of VN below 6 μM. Substitution of the yellow residues resulted in an IC_{50} for bis-ANS inactivation of PAI-1 in the presence of VN between 10 and 20 μM. Substitution of the cyan residues did not result in significant changes of the IC_{50} for bis-ANS inactivation of PAI-1 in the presence of VN. K325 is not visible with the orientation used. N169 is not visible because it is buried in the structure. The figures were prepared with SWISSPDBVIEWER v3.7b2 (http://www.expasy.ch).

Table 1
IC_{50} for neutralisation of PAI-1 by bis-ANS in the absence and the presence of VN

PAI-1 variant	Secondary structural element	IC_{50} without VN (μM)	IC_{50} with VN (μM)
wt		0.73 ± 0.06 (3)	51 ± 18 (6)
F100A	s2A	0.75 ± 0.05 (3)	2.7 ± 0.3 (3)
R103A	s2A–hE loop	0.63 ± 0.15 (3)	4.9 ± 2.5 (3)
L107A	s2A–hE loop	0.66 ± 0.13 (3)	5.6 ± 3.2 (3)
M112A	hE	0.73 ± 0.23 (3)	5.3 ± 1.7 (3)
T122A	s1A	0.60 ± 0.00 (3)	4.6 ± 2.4 (3)
K124A	s1A	0.71 ± 0.26 (3)	15 ± 2 (3)
Q125A	s1A	0.65 ± 0.11 (4)	4.4 ± 3.8 (3)
Q125K	s1A	0.68 ± 0.11 (3)	2.4 ± 1.0 (3)
E132A	hF	0.70 ± 0.20 (3)	12 ± 6 (6)
I137A	hF	0.67 ± 0.04 (3)	3.4 ± 1.4 (3)
N139A	hF	0.82 ± 0.10 (3)	4.6 ± 1.8 (3)
D140A	hF	0.66 ± 0.27 (3)	12 ± 4 (3)
W141	hF	0.71 ± 0.24 (3)	2.9 ± 0.5 (3)
M149A	hF–s3A loop	0.68 ± 0.04 (3)	13 ± 4 (3)
N152A	hF–s3A loop	0.67 ± 0.03 (3)	16 ± 3 (3)
R164A	s3A	0.65 ± 0.19 (3)	11 ± 4 (3)
K325A	s5A	0.60 ± 0.10 (3)	17 ± 1 (3)
N169A	s3A	0.55 ± 0.01 (3)	4.6 ± 1.9 (3)

The table shows means, standard deviations, and numbers of determinations. The following substituents did not differ from wt with respect to the IC_{50} values for bis-ANS neutralisation of PAI-1 in the absence or presence of VN: Q102A, D104A, K106A, Q109A, P113A, F116A, R120A, D127A, E130A, R133A, R135A, F136A, T144A, H145A, D160A, E283A, E315A.

PAI-1 in the preparations. The IC_{50} value for the bis-ANS-induced reduction of the specific inhibitory activity of PAI-1 is 50–100-fold higher for the PAI-1-VN complex than for PAI-1 alone [6–7]. Any PAI-1 variant with a defect in VN binding would therefore be expected to be less efficiently protected against bis-ANS by VN. The strategy chosen was therefore to measure the IC_{50} value for bis-ANS reduction of the specific inhibitory activity of PAI-1 variants in the absence and presence of VN.

In the absence of VN, the IC_{50} values for bis-ANS neutralisation were invariably around 0.7 μM (Table 1). In the presence of VN, wt and most variants had an IC_{50} value about 50 μM. Strikingly, however, the variants F100A, R103A, L107A, M112A, T122A, Q125A, Q125K, I137A, N139A, W141A, and N169A had an IC_{50} value below 6 μM in the presence of VN. In addition, the variants K124, E132A, D140A, M149A, N152A, R164A, and K325A had an IC_{50} value between 10 and 20 μM in the presence of VN.

4. Discussion

The 'protection against bis-ANS' assay used here has the advantage of measuring the interaction of VN with only the active PAI-1 in the preparations, without the risk of misinterpretations of the results due to a fraction of inactive, possibly latent PAI-1. VN protection against bis-ANS may be due to direct steric competition between VN and bis-ANS or to a VN-induced conformational change of the bis-ANS-binding area. Using charge reversal mutations, we previously implicated the region around R78, R117, and R120 in the binding of bis-ANS [6], suggesting a partial overlap between the VN and bis-ANS binding areas. Besides the binding affinity, IC_{50}-values are also sensitive to the rate by which the bis-ANS-induced, inactivation-associated conformational change spreads through PAI-1. Therefore, the protective effect of VN against bis-ANS may also depend on a slower bis-ANS-induced conformational change of the PAI-1-VN-complex than of free PAI-1. Generally, observation of a substitution having a different effect on the IC_{50} value for bis-ANS neutralisation of PAI-1 in the absence and presence of VN shows that the corresponding amino acid side chain is in different surroundings in PAI-1 in the absence and presence of VN.

In our analysis, we relied almost totally on A-substitutions (except for the Q125K substitution), of single, mostly surface-exposed residues. By avoiding charge reversal mutations, we circumvented the uncertainties related to the use of mutants with a changed bis-ANS affinity and were for instance able to analyse the importance of R120, although an R120E substitution leads to a reduced bis-ANS susceptibility ([6] and data not shown). Our conclusions of a lack of importance for VN binding of F116 and R120 is not in conflict with the report of a reduced VN affinity of the substituent F116A-K117A-L118A-F119A-R120A [12], as this extensive substitution, including that of the buried F119, may have perturbed the structure in a manner also involving the residues we have implicated in binding.

Among the residues implicated in VN–PAI-1 interactions here, F100, R103, L107, M112, T122, K124, Q125, I137, D140, and W141 are surface-exposed and localised in a restricted area between s2A, hE, and hF (Fig. 1). We propose that they form the surface of PAI-1 which binds the somatomedin B domain of VN. The present implication of Q125 in VN binding is in full agreement with the original conclusion by Lawrence et al. [11], reporting a reduced binding of PAI-1 Q125K. The fact that Q125 scores as necessary for VN protection against bis-ANS here, both when using a K and an A substitution, lends credibility to the present experimental strategy. According to the available three-dimensional structures of PAI-1 [8,17–20], the architecture of this region changes dramatically upon the stressed-to-relaxed transition due to the translocation of s1A and s2A, in agreement with relaxed PAI-1 having a much lower VN affinity than stressed PAI-1.

E132, N139, M149, N152, N169, R164, and K325, also implicated in VN–PAI-1 interactions here, are scattered outside the surface defined by the other residues (Fig. 1) and

therefore unlikely to be implicated directly in VN binding. Instead, these residues may be important for maintaining the proper architecture of the binding area or may reorient themselves upon VN binding and thereby confer a signal from the VN binding area to the RCL. In fact, most of these residues are localised at interfaces between secondary structural elements assumed to move relative to each other during RCL insertion (for a review, see [3]). An abnormal response to VN of the latency transition rate of K325A suggested that PAI-1 K325 plays an important role in conferring a signal from VN binding to the RCL [7,21].

Taken together, our observations suggest that VN binds in an area bordered by s2A, hE, and hF. By communication through hF, the hF–s3A loop, s3A, and s5A, this leads to conformational changes of the RCL. These changes in turn stabilise PAI-1 against latency transition, substrate behaviour, and polymerisation. More precise physico-chemical methods can now be used to define the exact role of each individual residue.

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