We have characterized the neutralization of the inhibitory activity of the serpin plasminogen activator inhibitor-1 (PAI-1) by a number of structurally distinct organochemicals, including compounds with environment-sensitive spectroscopic properties. In contrast to latent and reactive center-cleaved PAI-1 and PAI-1 in complex with urokinase-type plasminogen activator (uPA), active PAI-1 strongly increased the fluorescence of the PAI-1-neutralizing compounds 1-anilinonaphthalene-8-sulfonic acid and 4,4'-dianilino-1,1'-bisnaphthyl-5,5'-disulfonic acid. The fluorescence increase could be competed by all tested nonfluorescent neutralizers, indicating that all neutralizers bind to a common hydrophobic area preferentially accessible in active PAI-1. Activity neutralization proceeded through two consecutive steps as follows: first step is conversion to forms displaying substrate behavior toward uPA, and second step is to forms inert to uPA.

With some neutralizers, the second step was associated with PAI-1 polymerization. Vitronectin reduced the susceptibility to the neutralizers. Changes in sensitivity to activity neutralization by point mutations were compatible with the various neutralizers having overlapping, but not identical, binding sites in the region around α-helices D and E and β-strand 1A, known to act as a flexible joint when β-sheet A opens and the reactive center loop inserts as β-strand 4A during reaction with target proteinases. The defined binding area may be a target for development of compounds for neutralizing PAI-1 in cancer and cardiovascular diseases.

Plasminogen activator inhibitor-1 (PAI-1) is a fast and specific inhibitor of the serine proteinases urokinase-type (uPA) and tissue-type plasminogen activator (tPA) and, as such, an important regulator of extracellular proteolysis in turn over of extracellular matrix and in fibrinolysis (for reviews see Refs. 1 and 2). PAI-1 binds with high affinity to vitronectin (for reviews see Refs. 3 and 4) and may regulate cell migration and adhesion by inhibition of vitronectin binding of integrins and the uPA receptor (5–10). The PAI-1 level in malignant tumors is one of the most informative biochemical markers of a poor prognosis (for reviews see Refs. 11 and 12), and PAI-1 seems to be causally involved in tumor invasion and angiogenesis (13). A high PAI-1 level in blood plasma is a risk factor for ischemic cardiovascular disease and venous thromboembolism (for review see Ref. 14). PAI-1 is therefore a potential target for both anti-cancer and anti-thrombotic therapy.

PAI-1 belongs to the serpin superfamily. Serpins are composed of 3 β-sheets and 9 α-helices. Serpins and their target proteinases form stable complexes by interaction of the active site of the proteinases with the reactive center peptide bond (P1–P1') in the solvent-exposed, ~20-amino acid long peptide loop, the reactive center loop (RCL) (for reviews see Refs. 2 and 15–17). There is both structural and biochemical evidence that complex formation is associated with the P1–P1' bond being cleaved, the active site Ser of the proteinase linked to the carbonyl group of the P1 residue by an ester bond, the N-terminal part of the RCL inserted as strand 4 of the large central β-sheets A (s4A). The proteinase translocated across the plane of β-sheet A, toward the other pole of the molecule (18–27). Under specific in vitro conditions, however, the ester bond is hydrolyzed, and the serpin exhibits substrate behavior. This also leads to insertion of the N-terminal part of the RCL as s4A (for a review see Ref. 2). RCL insertion also takes place during transition to the inactive, latent state, occurring spontaneously only in PAI-1 (28). Latent PAI-1 can be reconverted to the active form by denaturation and refolding (29).

During RCL insertion, the so-called small serpin fragment, consisting of s1A, s2A, s3A, α-helix F (hF) and the loop connecting hF and s3A (the hF/s3A-loop), moves relative to the rest of the molecule, the large serpin fragment, to make space for the RCL between s3A and s5A. The regions around hD and hE were proposed to form a flexible joint during RCL insertion (30) (Fig. 1). The idea of flexibility of this region was supported by the observation that it contains a cluster of peptide bonds with differential susceptibility to nontarget proteinases in active, latent, and reactive center-cleaved PAI-1 (31). Comparison of the x-ray crystal structures of latent, active, and reactive plasminogen activator; wt, wild type; CHO, Chinese hamster ovary; RCL, reactive center loop; PAGE, polyacrylamide gel electrophoresis.

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Latent PAI-1 was converted to the active form by denaturation in 4 mM guanidinium chloride and refolding by extensive dialysis against phosphate-buffered saline (PBS; 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl) at 0 °C. Active PAI-1 was stored at −80 °C. Reactive center-cleaved PAI-1 (form C) and uPA:PAI-1 complex were prepared as described (31).

**Conformational Changes**

Human PAI-1 was purified in the latent form from serum-

\[ F = \frac{F_{\text{obs}}}{F_{\text{m}}} = \frac{[\text{ANS}]}{[\text{ANS}]_{\text{B}}}, \quad \text{where} \quad [\text{ANS}]_{\text{B}} = \frac{[\text{ANS}]}{[\text{ANS}]_{\text{obs}}} \quad \text{and} \quad [\text{ANS}]_{\text{obs}} \quad \text{are the corrected and the observed fluorescence intensities, respectively}. \]

The concentration of free bis-ANS, [bis-ANS], was calculated by subtracting [bis-ANS] from the total bis-ANS concentration. Assuming a simple binding equilibrium, one arrives at Equation 1,

\[ [\text{bis-ANS}] = \frac{F_{\text{m}}}{F} \]  

**Fluorescence Measurements**

Fluorescence emission spectra for ANS and bis-ANS in the absence and presence of PAI-1 were recorded with a SPM 25 spectrophluorimeter (Kontron Instruments), using excitation wavelengths of 386 and 395 nm, respectively. The emission was recorded over the range of 400–600 nm. The change in fluorescence was measured after 10 min mixing PAI-1 with the fluorescent ligands in a buffer of 0.1 M Tris-HCl, pH 8.1, at 14 °C, unless otherwise indicated.

The observed fluorescence intensities, 

\[ [\text{bis-ANS}]_{\text{obs}} \quad \text{and} \quad [\text{ANS}]_{\text{obs}} \quad \text{are the corrected and the observed fluorescence intensities, respectively} \]

**Estimation of the dissociation constants for nonfluorescent neutralizers**

The corrected for the inner filter effect were performed using the equation

\[ F = \frac{F_{\text{obs}}}{F_{\text{m}}} = \frac{[\text{ANS}]}{[\text{ANS}]_{\text{B}}}, \quad \text{where} \quad [\text{ANS}]_{\text{B}} = \frac{[\text{ANS}]}{[\text{ANS}]_{\text{obs}}} \quad \text{and} \quad [\text{ANS}]_{\text{obs}} \quad \text{are the corrected and the observed fluorescence intensities, respectively}. \]

**Estimation of the dissociation constants for nonfluorescent neutralizers**

The concentration of free bis-ANS, [bis-ANS], was calculated by subtracting [bis-ANS] from the total bis-ANS concentration. Assuming a simple binding equilibrium, the dissociation constant, \( K_d \), for bis-ANS:PAI-1 binding was obtained by measuring the fluorescence intensity at 470 nm at several ANS concentrations. Assuming a simple binding equilibrium, one arrives at Equation 3,

\[ \text{RFI}_1 = \frac{[\text{ANS}] \times K_d}{[\text{ANS}]^2 + [\text{ANS}]_p} \]

Therefore, \( K_d \) could be determined by nonlinear regression analysis of plots of RFI1 versus the [ANS] values on the basis of Equation 3.

**Estimation of the dissociation constants for nonfluorescent neutralizers**

The concentration of free bis-ANS, [bis-ANS]p, was calculated by subtracting [bis-ANS] from the total bis-ANS concentration. Assuming a simple binding equilibrium, the dissociation constant, \( K_d \), for bis-ANS:PAI-1 binding was obtained by measuring the fluorescence intensity at 470 nm at several ANS concentrations. Assuming a simple binding equilibrium, one arrives at Equation 3,

\[ \text{RFI}_1 = \frac{[\text{ANS}] \times K_d}{[\text{ANS}]^2 + [\text{ANS}]_p} \]

Therefore, \( K_d \) could be determined by nonlinear regression analysis of plots of RFI1 versus the [ANS] values on the basis of Equation 3.

**Experimental Procedures**

**PAI-1 Neutralization**

PAI-1—PAI-1 residues were numbered by the \( \alpha_1 \)-proteinase inhibitor (\( \alpha_1 \)-PI) template numbering system, based on the alignment of PAI-1 and \( \alpha_1 \)-PI by Hober and Carroll (15).

Natural human PAI-1 was purified in the latent form from serum-free conditioned medium of dexamethasone-treated HT-1080 cells by immunoaffinity chromatography (43). Amino acid sequencing of the protein gave the expected, slightly modified N-terminal sequence MHVHPPSYVAHL. Expression of recombinant wt and mutant PAI-1 in the yeast Pichia pastoris and its purification from the conditioned medium in the latent form were performed as described (52).

Human wt and PAI-1 R97E/R136E/R139E were expressed in CHO cells as before (50).
PAI-1 Neutralization

...ers was done by competition studies, in which 0.5 or 1 μM PAI-1 was preincubated with ANS (100 μM), and the fluorescence intensity at 470 nm was then recorded 10 min after addition of nonfluorescent competitors in various concentrations. The fluorescence intensity at λmax was expressed relative to that obtained in the absence of nonfluorescent competitor (FL). Assuming competition of fluorescence inhibition by specific ligands for binding to one site, an excess of nonfluorescent ligand I over PAI-1, and simple binding equilibria for both ANS and I, the following Equation 4 is true.

\[
RFL = (K_r + [ANS]I) / [K_r + [ANS] + (K_s/K_r) \cdot [I]_r) \tag{Eq. 4}
\]

K_r is the dissociation constant for I-PAI-1 binding; K_s the dissociation constant for ANS-PAI-1 binding; [ANS]I the total ANS concentration; and [I]_r the total I concentration. Thus, the K_s values were obtained by nonlinear regression analysis of plots of RFL versus [I]_r on the basis of Equation 4, the K_r for ANS binding having been determined in advance.

Measurements of the Effects of Neutralizers on Specific Inhibitory Activity of PAI-1—The specific inhibitory activity of PAI-1, i.e., the fraction of the total amount of PAI-1 forming a stable complex with uPA at the conditions used, was measured by titration of PAI-1 against uPA in a peptidyl anilide assay, in the presence of several concentrations of each neutralizing compound. Shortly, PAI-1 was serially diluted with a buffer of 0.1M Tris-HCl, pH 8.1, 0.25% gelatin at 37 °C, corresponding to concentrations between 0.02 and 40 μg/ml and a volume of 100 μl. To each dilution series, a particular neutralizing compound was added in a particular concentration and incubated with PAI-1 for 10 min at 37 °C. Portions of uPA solutions of the same temperature and in the same buffer were then added, corresponding to a final uPA concentration of 0.25 μg/ml. The final volume of 200 μl was correspondingly adjusted to 0.02 and 20 μg/ml. Incubation was then continued until the process of inhibition of uPA had come to an end. Control experiments showed that this was in all cases achieved in less than 2 min. The remaining uPA enzyme activity was determined by incubation with the peptidyl anilide substrate S-2444 at 37 °C 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 0.25 μg/ml uPA. The IC50 values for the compounds being tested, i.e., the concentrations causing a 50% reduction in the specific inhibitory activity against the concentration of the compounds in the final assay mixture.

Measurement of Time Course of Changes in the Specific Inhibitory Activity of PAI-1—PAI-1 was incubated at various concentrations between 0.33 and 330 μg/ml in 0.1 M Tris-HCl, pH 8.1, 0.25% gelatin, at 0 or 37 °C, without or with neutralizers. After various times, samples were removed for assay of specific inhibitory activity, which in this case was performed by diluting all samples to a final PAI-1 concentration of 0.33 μg/ml final volume of 200 μl and the same temperature as the original incubation. uPA was added to a final concentration equivalent to 90% of the activity of PAI-1 in the absence of neutralizers. The uPA solutions were without or with BSA in a concentration corresponding to a final concentration, in gels with 6–16% polyacrylamide. The gels were stained with Coo-...
and free ligand. By analysis of the relationship between bound and free bis-ANS, we found a $K_d$ of $0.57 \pm 0.29 \mu M$ and a number of binding sites per PAI-1 molecule of $1.66 \pm 0.62$ ($n = 12$) (Fig. 4B and Table I).

With ANS, only a small fraction of the ligand added to $\mu M$ concentrations of PAI-1 was bound to the protein, rendering calculations of the stoichiometry impossible. However, an estimate of the $K_d$ was obtained by studying the ANS concentration dependence of the fluorescence intensity in the presence of PAI-1 (Table I).

To measure the dissociation constants for the binding to PAI-1 of the nonfluorescent neutralizers, PAI-1 was preincubated with 100 $\mu M$ ANS, and the fluorescence intensity was recorded after addition of increasing concentrations of the nonfluorescent compounds. AR-H029953XX, 1-dodecyl sulfuric acid, or XR5118 caused a decrease in the ANS fluorescence intensity down to background levels. In contrast, two non-neutralizing, nonfluorescent compounds, 1-nonanesulfonic acid and 2-propylpentanoic acid, caused very little inhibition of the fluorescence in concentrations up to 1 mM (Fig. 5).

$m$ values for the nonfluorescent compounds were determined by analysis of the displacement curves (Table I).

Similar competition experiments were done with bis-ANS. The results obtained (not shown) were in full agreement with those obtained with ANS.

The fluorimetrically determined dissociation constants for ANS, bis-ANS, 1-dodecyl sulfuric acid, and XR5118 (Table I)
were not significantly different from the corresponding IC₅₀ values (Fig. 2). Only in the case of AR-H029953XX, the dissociation constant was somewhat lower than the IC₅₀ value. This disagreement may be related to the irreversible reaction of PAI-1 with uPA leading to dissociation of AR-H029953XX/PAI-1 complex during the determination of PAI-1-specific inhibitory activity.

The results of the fluorimetric analyses are most readily explained by the hypothesis that all the tested neutralizers have overlapping binding sites. The five neutralizers were tested on a series of PAI-1 variants, expressed in P. pastoris or CHO cells (Table II). The IC₅₀ values for neutralization of P. pastoris and CHO PAI-1 wt were not significantly different from those for neutralization of HT-1080 PAI-1. The susceptibility to the neutralizers was changed by substitution of specific amino acids in the flexible joint region. The P94K substitution, in hD (see Fig. 1), caused a 10-fold increase in the IC₅₀ for XR5118 but had no effects on the IC₅₀ for the negatively charged neutralizers. The double substitution R97K/H98K was without effect. The triple substitution R97E/R136E/R139E (see Fig. 1) increased the IC₅₀ value for AR-H029953XX from about 5 μM to more than 20 μM and increased the IC₅₀ values for ANS-, bis-ANS-, and 1-dodecyl sulfuric acid neutralization about 3-fold, but did not affect the IC₅₀ value for XR5118 neutralization. Conclusively, the IC₅₀ values for all neutralizers were sensitive to mutations in the flexible joint region, consistent with the conclusion from the fluorimetric measurements of these compounds having overlapping binding sites. Still, the substitutions in the flexible joint region affecting the susceptibility to the positively charged neutralizers had no effect on the susceptibility to the negatively charged neutralizer and vice versa.

### Dissociation constants for binding of neutralizers to PAI-1

The dissociation constants were determined either as IC₅₀ values by analysis of the fluorescence increase associated with the binding of ANS and bis-ANS to HT-1080 PAI-1 or as Kᵣ values by analysis of the competition of ANS binding to HT-1080 PAI-1 by the nonfluorescent compounds (AR-H029953XX, 1-dodecyl sulfuric acid, 1-nonanesulfonic acid, 2-propylpentanoic acid, and XR5118). Further details are given in the text. The data are given as means, S.D., and numbers of experiments.

| Compound                  | Dissociation constant μM |
|---------------------------|--------------------------|
| ANS                       | 78 ± 21 (4)              |
| AR-H029953XX              | 0.49 ± 0.15 (4)          |
| Bis-ANS                   | 0.57 ± 0.29 (12)         |
| 1-Dodecyl sulfuric acid   | 24 ± 8 (4)               |
| 1-Nonanesulfonic acid     | >1000 (1)                |
| 2-Propylpentanoic acid    | >1000 (1)                |
| XR5118                    | 2.7 ± 1.7 (4)            |

The parameters of the curves drawn were obtained by nonlinear regression analysis of the data according to Equation 4, using a $K_r$ value for ANS binding of 78 μM (see Table I) and [ANS]₀ = 100 μM. The estimated $K_r$ values in the experiments shown are as follows: AR-H029953XX, 0.65 μM; 1-dodecyl sulfuric acid, 33 μM; XR5118, 3.9 μM. The results of several experiments with each compound are summarized in Table I.
Preincubation of PAI-1 with vitronectin decreased its susceptibility to all five neutralizers but to a variable extent. Thus, the IC \(_{50}\) value for bis-ANS increased about 50-fold, to 27 \(\mu\)M (\(n = 3\)), but the IC \(_{50}\) value for 1-dodecyl sulfuric acid increased only about 7-fold, to 110 \(\mu\)M (\(n = 4\)). The IC \(_{50}\) value for XR5118 increased to more than 250 \(\mu\)M and that for ANS to more than 1000 \(\mu\)M.

**Time Course of Changes of PAI-1-specific Inhibitory Activity**—The PAI-1-specific inhibitory activity was determined after incubations for various times at 0 or 37 °C, at various PAI-1 concentrations, without or with neutralizers in concentrations of severalfold the IC \(_{50}\) values and severalfold the PAI-1 concentrations, without or with neutralizers in concentrations of severalfold the IC \(_{50}\) values and severalfold the PAI-1 concentrations.

| Neutralizer          | P. pastoris PAI-1 variant | CHO PAI-1 variant |
|----------------------|---------------------------|-------------------|
|                      | wt                        | P94K              | R97K/H98K                   | wt                        | R97E/R136E/R139E |
| ANS                  | 116 ± 59 (19)             | 101 ± 6 (3)       | 62 ± 30 (7)                 | 82 ± 14 (3)               | 256 ± 96 (5)       |
| AR-H029953XX         | 5.0 ± 0.6 (3)             | 5.4 ± 0.7 (3)     | NDb                          | 6.4 ± 5.9 (3)             | >20 \(\mu\)M       |
| Bis-ANS              | 0.64 ± 0.08 (6)           | 0.93 ± 0.12 (3)   | 0.63 ± 0.09 (3)             | 0.79 ± 0.18 (3)           | 3.1 ± 0.5 (3)*     |
| 1-DS                 | 22 ± 8 (8)                | 22 ± 3 (3)        | 19 ± 4 (3)                  | 20 ± 9 (3)                | 49 ± 4 (3)*        |
| XR5118               | 16 ± 8 (4)                | 151 ± 54 (3)a     | 22 ± 4 (3)                  | 5.2 ± 1.4 (3)             | 4.8 ± 1.2 (3)      |

* Statistically significant from PAI-1 wt (\(p < 0.005\)).  

**TABLE II**

Effect of PAI-1 neutralizers on PAI-1 variants

The IC \(_{50}\) values (\(\mu\)M) for the effect of the neutralizers on the specific inhibitory activities of the PAI-1 variants expressed in *P. pastoris* or CHO cells were estimated. The data given are means and S.D. from the indicated numbers of experiments. 1-DS, 1-dodecyl sulfuric acid.

The time course of the activity loss induced by the positively charged XR5118 was not significantly different in assays without and with BSA, showing that the XR5118-induced activity loss was totally irreversible. The rate of XR5118-induced activity loss was strongly temperature-dependent. We found no evidence that the rate of XR5118-induced neutralization in-
increased with increasing PAI-1 concentration, suggesting that the neutralization process proceeded monomERICALLY (data not shown).

**Analysis of the Functional Behavior of Neutralizer-induced Forms of PAI-1 by Reaction with LMW-uPA and SDS-PAGE**—

The effect of the neutralizers on the functional behavior of PAI-1 was analyzed by reacting neutralizer-treated PAI-1 with a molar excess of LMW-uPA and separating the reaction products by SDS-PAGE. A representative experiment, with bis-ANS, is shown in Fig. 7. Without incubation with neutralizers, most of the PAI-1 formed a stable complex with LMW-uPA, whereas minor fractions were latent or exhibited substrate behavior, respectively (Fig. 7, lane to the left). Complex formation was totally abolished within 2 min of incubation of PAI-1 with bis-ANS at 37 °C. An inactive form with substrate behavior predominated for the first 2 min, after which time PAI-1 was gradually converted to an inert form. At 0 °C, complex formation was abolished more slowly, and substrate behavior predominated for at least 2 h (data not shown). Identical observations were done with ANS, AR-H029953XX, and 1-dodecyl sulfuric acid. Neutralization by the positively charged XR5118 was at 0 °C associated with a fast conversion to a form with a substrate behavior, followed by conversion to an inert form, but at 37 °C, there was no detectable increase in substrate behavior, but an immediate increase in the amount of the inert form (data not shown). HT-1080 PAI-1 and *E. coli* PAI-1 reacted in the same manner to all neutralizers (data not shown).

**Characterization of Neutralizer-induced PAI-1 Forms by Gel Filtration**—PAI-1 was preincubated at 0 or 37 °C in the absence or presence of neutralizers, for practical reasons with an LMW-uPA concentration of 330 μg/ml for 30 min at 37 °C without or with neutralizers (Fig. 11). Without neutralizers, PAI-1 migrated mainly as a monomer in the expected position relative to the Mr markers. With the negatively charged neutralizers, but not with XR5118, most PAI-1 was converted to distinct, slower migrating bands. By comparison to the migration of the marker, the PAI-1 bands seem to represent dimers, trimers, tetramers, etc. The gel system did not allow resolution of polymer species with an Mr above ~300,000. We concluded that PAI-1 neutralization by the negatively charged neutralizers, but not XR5118, is associated with formation of distinct PAI-1 polymers.

**A Two-step Neutralization Mechanism**—Comparing the time course of the change of PAI-1-specific inhibitory activity (Fig. 6), the time course of the changes in the functional behavior (Fig. 7), the time course of polymerization (Fig. 8), and the functional behavior of individual peaks of the gel filtration profiles (Fig. 10), we concluded that PAI-1 neutralization follows variations over a basic two-step mechanism, by which neutralizer-complexed PAI-1 (N−PAI-1) is rapidly converted to a form exhibiting substrate behavior (N−PAI-1S) and subsequently to an inert form (N−PAI-1I):

\[
N−PAI-1→N−PAI-1S→N−PAI-1I
\]

With the negatively charged neutralizers AR-H029953XX, bis-ANS, and 1-dodecyl sulfuric acid, the first step is reversible upon removal of the neutralizer, as evidenced by the recovery of specific inhibitory activity in assays with BSA (Fig. 6) and the reappearance of inhibitory activity in the monomer peak of the gel filtration profile (Fig. 10), whereas the second step consists of an irreversible polymerization, as evidenced by the absence of inhibitory activity of the polymer peaks of the gel filtration profiles and the PAI-1 concentration dependence of the rate of loss of specific inhibitory activity in assays with BSA (Fig. 6). The fact that the time course of the irreversible neutralization by ANS was independent of the PAI-1 concentration suggests the existence of an intermediate, irreversibly inactivated monomeric form of ANS-complexed PAI-1, which subsequently polymerizes. XR5118-induced neutralization seemed to proceed by two irreversible conversions, neither of which involves polymerization.
Fifty-μg portions of HT-1080 PAI-1, in active form, were subjected to gel filtration. Conditions of preincubation of PAI-1 (in a concentration of 330 μg/ml or 6 μM in PBS) before the gel filtration are indicated on each elution profile. The portions were then subjected to gel filtration on a Superdex 200 HR10/30 column in a buffer of 0.1M Tris-HCl, pH 8.1, 0.5 M NaCl. The migration of the marker proteins BSA (Mr 67,000), murine IgG (Mr 150,000), and β-galactosidase (Mr 540,000) are indicated by arrows above the profiles. V_0, void volume; no add., no addition; 1-DS, 1-dodecyl sulfuric acid.

**DISCUSSION**

On the basis of the fluorimetric binding assays and the site-directed mutagenesis studies reported here, we concluded that four negatively charged (ANS, AR-H029953XX, bis-ANS, and 1-dodecyl sulfuric acid) and one positively charged (XR5118) organochemical PAI-1 neutralizers have overlapping but not identical binding sites in a hydrophobic area in the flexible joint region of PAI-1. Neutralization proceeds through two steps as follows: first conversion to forms with substrate behavior and second to inert forms. With the negatively charged neutralizers, but not the positively charged one, the second step was associated with PAI-1 polymerization.

Besides the five neutralizers studied in detail, a number of other amphipathic organochemicals also neutralized PAI-1 (Fig. 2). Some of the compounds, including 1-dodecyl sulfuric acid and deoxycholic acid, are generally used as detergents. However, the neutralizing effect of the five compounds studied in detail did not seem to depend on their detergent properties. First, the critical micelle concentration for 1-dodecyl sulfuric acid is 8 mM (60, 61), much higher than its 15 μM IC\(_{50}\) value for PAI-1 neutralization. Second, although structurally very different compounds were able to neutralize PAI-1 with a relatively low IC\(_{50}\) value, a certain specificity was observed. For instance, among the two carboxylic acids with unbranched aliphatic side chains, the length of the side chain appeared to be decisive for activity (Fig. 2). Third, the observations that XR5118 inhibited the polymerization induced by the negatively charged neutralizers (Fig. 9) are difficult to reconcile with a detergent mechanism. Fourth, the well defined PAI-1 polymers observed by native gel electrophoresis (Fig. 11) are in contrast to the expectancies from a detergent-induced, nonspecific aggregation.

Rather, our findings point to the five neutralizers exerting their effect by binding to overlapping sites in a hydrophobic area with a relatively low binding specificity. This notion is supported by the observation that all nonfluorescent neutralizers competed the binding of ANS and bis-ANS (Fig. 5) and by the largely good agreement between the IC\(_{50}\) values (Fig. 2) and the fluorimetrically determined dissociation constants (Table I). Likewise, our bis-ANS binding studies were compatible with a single class of binding sites with respect to binding affinity. Although the binding studies led to determination of the number of bis-ANS-binding sites per PAI-1 molecule to a value between 1 and 2 and therefore did not exclude the existence of two independent bis-ANS-binding sites, the induction of about 80% polymerization of 6 μM PAI-1 by 5 μM bis-ANS (Fig. 8) is a strong argument for a single binding and effector site for bis-ANS. Furthermore, the observed XR5118 inhibition of the polymerization induced by the negatively charged neutralizers (Fig. 9) is most readily explained by XR5118 and the negatively charged neutralizers having overlapping binding sites. Thus, it seems most likely that both the reversible and irreversible neutralizer effects are caused by binding of one neutralizer molecule per PAI-1 molecule. The possibility of several effector sites in PAI-1, each causing neutralization by a specific mechanism, seems remote.

It was previously suggested that the flexible joint region of PAI-1 contains a regulatory, hydrophobic, ligand-binding area (50). The x-ray crystal structures of active PAI-1 and \(\alpha_1\)PI are in agreement with a hydrophobic cavity in this area (33, 34, 62,
Our present findings are consistent with the hypothesis that the five neutralizers bind in this cavity. First, amino acid substitutions in hD and hE changed the IC50 values. Second, the stronger binding of ANS and bis-ANS to active than to latent, reactive center-cleaved and uPA-complexed PAI-1 is in agreement with the expectancies from the x-ray crystal structures, showing that the distance between s2A and hD and hE decreases upon insertion of RCL as s4A (28, 32–34, 40). The volume of the hydrophobic cavity in active PAI-1 was estimated to 676 Å³ (63). Each of the bulky and in most cases rigid neutralizer molecules would therefore be expected to occupy a large fraction of the volume of the cavity. Thus, the binding competition between the negatively and the positively charged neutralizers is not in conflict with the observation that the amino acid substitutions in hD and hE, respectively, affected the IC50 values for the negatively and the positively charged neutralizers differently. This observation is, in fact, in full agreement with their different structures and the charge changes associated with the substitutions. We therefore suggest that the variation in neutralization kinetics, in the induced molecular changes, and in the differential response to vitronectin is due to different neutralizers occupying different subsites within the same hydrophobic area. In this way, the different neutralizers may have different effects on the conformation of β-sheet A and thereby different effects on the movements of the RCL and the tendency to polymerization. This situation is reminiscent of estrogen receptor binding of estrogen agonists, antagonists, and partial agonists, which have overlapping binding sites, but induce different conformational changes in the receptor protein (64–66).

Induction of serpin polymerization by small organochemical ligands is a novel finding. Previously described serpin polymerization all implicate the RCL of one serpin molecule forming an additional strand in β-sheet A or β-sheet C of another molecule. There is evidence for three different modes of loop-sheet polymerization. 1) Heating or mutations in the so-called shutter region can lead to polymerization by the RCL of one molecule inserting as s4A of another molecule (for reviews see Refs. 2 and 17). 2) The RCL of one molecule can insert as s1C in another molecule, in which the intrinsic s1C has been extracted (67–69). 3) The RCL of one molecule can hydrogen-bond to s6A of another molecule and thus form an s7A (33, 34). It is striking that the fluorescence observed by addition of ANS and bis-ANS to active PAI-1 remained high after incubation under conditions leading to polymerization, whereas the fluorescence induced by addition of these compounds to latent, reactive center-cleaved and uPA-complexed PAI-1 was much lower. This observation argues against the polymerization involving insertion of the RCL as s4A and favors the other modes of polymerization. The mode of the polymerization described here is clearly different from the recently reported PAI-1 polymerization induced in both latent and active PAI-1 at the strongly acidic pH of 4 (70).

An important perspective is the possibility of utilizing the hydrophobic area as a target for anti-cancer and anti-thrombotic drugs. To do so, strategies must be developed to circumvent certain problems that have become apparent from the studies described here. First, the strong binding to serum albumin common to all the compounds studied here must be avoided. Second, since PAI-1 is expected to be bound to

FIG. 10. Analysis of 1-dodecyl sulfuric acid-induced PAI-1 forms by gel filtration and SDS-PAGE. HT-1080 PAI-1 (330 µg/ml) was incubated with 100 µM 1-dodecyl sulfuric acid for 30 min at 0 or 37 °C, as indicated. Two hundred-µg portions were then subjected to gel filtration. The absorbance at 280 nm is shown versus the elution time. Five-µg portions of PAI-1, either before gel filtration or from individual peaks of the gel filtration profile, were reacted with 10 µg of LMW-uPA for 2 min at 37 °C and then subjected to SDS-PAGE. The positions of LMW-uPA:PAI-1 complex, native/inert PAI-1, reactive center-cleaved PAI-1 (RCC-PAI-1) and LMW-uPA are indicated to the left.

FIG. 11. Characterization of neutralizer-induced PAI-1 forms by native gel electrophoresis. Ten-µg portions of HT-1080 PAI-1 (330 µg/ml) were incubated for 30 min at 37 °C in the absence or presence of neutralizers, as indicated. They were then subjected to native polyacrylamide gel electrophoresis. The migrations of the marker proteins are indicated to the left.
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vitronectin in vivo, pharmacologically potentially interesting molecules must have a high affinity not only to free PAI-1 but also to PAI-1 in its vitronectin-associated state. Third, other serpins have similar hydrophobic areas, and the specificity of PAI-1 neutralizers of potential interest for in vivo use must therefore be ensured.

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A Regulatory Hydrophobic Area in the Flexible Joint Region of Plasminogen Activator Inhibitor-1, Defined with Fluorescent Activity-neutralizing Ligands: LIGAND-INDUCED SERPIN POLYMERIZATION

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