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Immunological discrimination of diverse forms of human α_1-proteinase inhibitor*

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The immunodiffusion cross-reactivity and competitive inhibition ELISA assays were used for immunological differentiation of latent form, cleaved form and guanidinium hydrochloride (GuHCl) induced polymer of human α_1-proteinase inhibitor (α_1-PI). Under the conditions studied, the differences between latent form and GuHCl-induced polymers of the inhibitor in terms of immunological response were estimated to amount to about 30% and differences between latent and cleaved α_1-PI to about 50%. The immunodiffusion and ELISA data for citrate-induced polymers suggest that in their structure the latent molecule is involved.

On the basis of competitive inhibition data, we suggest that the α_1-PI protein polymerisation involves insertion of the reactive-site loop (RSL) into the A-sheet under mild conditions and that in the latent form of the inhibitor RSL is incompletely inserted into the A-sheet.

Serpins constitute a superfamily of glycoproteins, many of which are inhibitors of serine proteinases. Human α_1-proteinase inhibitor (α_1-PI) is the best known member of the family. It has a typical structure of serpin with 30% formed by helices and 40% by three β-sheets. One of these sheets, the five-stranded A-sheet, characterises the intact molecule with the reactive-center peptide loop exposed, whereas the six-stranded A-sheet is present in a cleaved form of α_1-PI. It is widely assumed that the additional strand of A-sheet of the cleaved molecule results from insertion of a protruded reactive loop occuring on proteolysis [1]. Recent reports concerning several serpins have confirmed that the reactive-site loop (RSL) is mobile and able to adopt varying conformations [2, 3]. The location of RSL depends on particular conformation of α_1-PI, and RSL can exist as a fully exposed loop, or be fully integrated into the center of the major β-sheet, or have an intermediate location. These locations of the RSL can be induced by various mild denaturing conditions, e.g. heating of the inhibitor in the presence of 0.7 M sodium citrate at 67°C induces the so called latent form with the RSL inserted into the A-sheet [4]. On the other hand, when the protein is exposed to 1 M

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Abbreviations: α_1-PI, α_1-proteinase inhibitor; ATIII, antithrombin III; BSA, bovine serum albumin; GuHCl, guanidinium hydrochloride; PBS, phosphate buffered saline; PBST, PBS + Tween; RSL, reactive site loop.

guanidinium hydrochloride (GuHCl) at 4°C for 24 h formation of long polymers is observed whereas a shorter incubation time at 37°C favours formation of the short chain polymers [5]. The polymers of α_1-PI can be also formed when the native inhibitor is heated for several hours at 48°C [6].

The polymerisation phenomena of α_1-PI have a clinical significance in explaining the secretion defect of the Z-mutant of the inhibitor. This defect results in vastly decreased concentration of circulating α_1-PI due to polymerisation and can contribute to the development of emphysema. Polymerisation is responsible for α_1-PI accumulation as amorphous deposits in endoplasmatic reticulum of hepatocytes of affected individuals [7].

In this paper the immunological evidence for differentiation of GuHCl- and citrate-induced polymers, and latent and cleaved forms of α_1-PI is presented.

MATERIALS AND METHODS

Preparation of antigens and antisera. Human α_1-PI purified by the method of Bruch & Bieth [8] was cleaved at Glu354-Ala355 by V8 proteinase as described elsewhere [9].

Polymerisation of α_1-PI was induced by incubation of the native inhibitor (2 mg/ml) in the presence of 1 M GuHCl in 50 mM Tris, 50 mM KCl and 0.1% (v/v) β-mercaptoethanol, pH 7.4, buffer for 20 h at 4°C followed by overnight dialysis against the same buffer [2]. Polymerisation was monitored by means of measurements with Static Laser Scattering Spectrophotometer SLS-700 (Otsuka Electronics Co.) at 632.7 nm (Fig. 1). The α_1-PI polymer sample with apparent molecular weight of about 500000 was taken as antigen for the ELISA and double diffusion experiments.

The latent form of α_1-PI was induced by incubation of native α_1-PI with 0.7 M sodium citrate at 67°C for 16 h and separated on Mono-Q column according to Lomas *et al.* [4]. This form demonstrated a characteristic cathodal shift compared to the native inhibitor as reported by Lomas *et al.* [4]. Another fraction identified by means of non-denaturing PAGE as short chain polymers, further refferd to as citrate-induced polymers, was used as an antigen in double diffusion and ELISA experiments.

Rabbits (Japanese White) were immunised using multisite subcutaneous injections. Antiserum against the polymers and cleaved forms of α_1-PI was produced by emulsifying the antigen at a concentration of 0.5 mg/ml diluted in phosphate buffered saline (PBS) with an equal volume of Freund's complete adjuvant, and 2 ml of the emulsion was injected into each rabbit. Booster injections, using incomplete adjuvant, was applied twice at two-week intervals, with the antigen concentration of 1 mg/ml. The rabbits were bled two weeks after the last injection.

The antibodies were absorbed quantitatively with intact α_1-PI according to Wallgren *et al.* [10]. The absorption was conducted at the antigen concentration determined from a precipitation curve. The immunodiffusion experiments were carried out on 1% agarose gel on a glass plate in 50 mM Tris, 0.1 M sodium chloride, pH 7.4 [11].

Competitive inhibition assay. The enzyme-linked immunosorbent assay (ELISA) measurements were performed on the MaxiSorp plates from Nunc (Denmark). Antigens (1 mg/ml) in a PBS were incubated overnight at 4°C in 96-well plates. The binding of antigens to the plates was checked by the bicinchoninic acid procedure [12]. All the studied antigenic forms bind equally within experimental errors to the plastic material. The plates were preincubated with 1% bovine serum albumin-PBS-0.05% Tween 20 solution (1% BSA-PBST) for 30 min at 37°C to prevent non-specific binding and washed with PBS-0.05% Tween (PBST). A rabbit antiserum, diluted 40000 fold with 1% BSA-PBST solution, was then incubated with the antigens for 2 h at 37°C. Then sheep anti-rabbit IgG horseradish peroxidase conjugates (POD-anti-rabbit sheep IgG, Cappel Oregon Teknika Co., West Chester, PA, U.S.A.) diluted 80000 fold with the same solution, were added and incubated for another hour at 37°C. The above conditions met the requirements of linear response in terms of antigen and antiserum concentration (not shown). After each incubation with antibodies, the plates were washed three times with PBST. The bound conjugate was allowed to react with chromogen (o-phenyldiamine at 0.4 mg/ml in 50 mM citrate/phosphate buffer, pH 5.0) for 30 min. The reaction was stopped by adding 25 ml of 2.5 M sulfuric acid, and absorbance of each well was determined with a Micro-ELISA reader at 492 nm.

For competitive inhibition assay, the antiserum raised against the GuHCl-induced polymers of α_1-PI and absorbed with intact or cleaved inhibitor was incubated with different concentrations of the polymers or cleaved form, as competitors, overnight at 4°C. Then it was centrifuged, diluted 40000 fold, and used for the competitive binding of antibodies to the antigens attached to a solid phase. The antibody-antigen reaction was normalised by setting the maximum of absorbance for each antigen to 100% at zero competitor concentration. The background of non-specific binding was determined by coating the wells with 1% BSA-PBST solution instead of antigen. A preimmunized rabbit serum was used as a control. The absorbance of preimmune serum with the antigens under the conditions studied did not exceed a 0.05 value.

The ELISA measurements of citrate-induced polymers and latent form of α_1-PI were performed with the whole antiserum raised against cleaved inhibitor and the antiserum was not preincubated with any antigens.

RESULTS

Different forms of α_1-PI were analysed by immunodiffusion and the ELISA with the antiserum raised against the GuHCl-induced

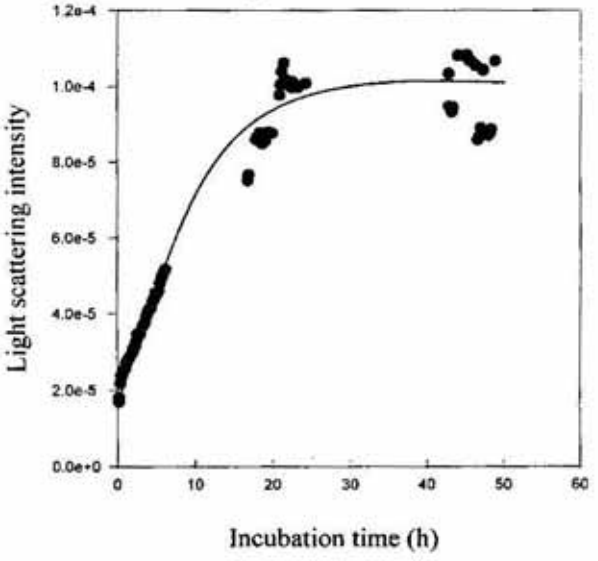

Fig. 1. Laser scattering light intensity plot of α_1-PI versus incubation time at 45°C.
The protein sample was diluted in 1 M guanidinium hydrochloride and concentration of the inhibitor was equal to 2 mg/ml. Scattering light intensities were monitored at 637.2 nm.

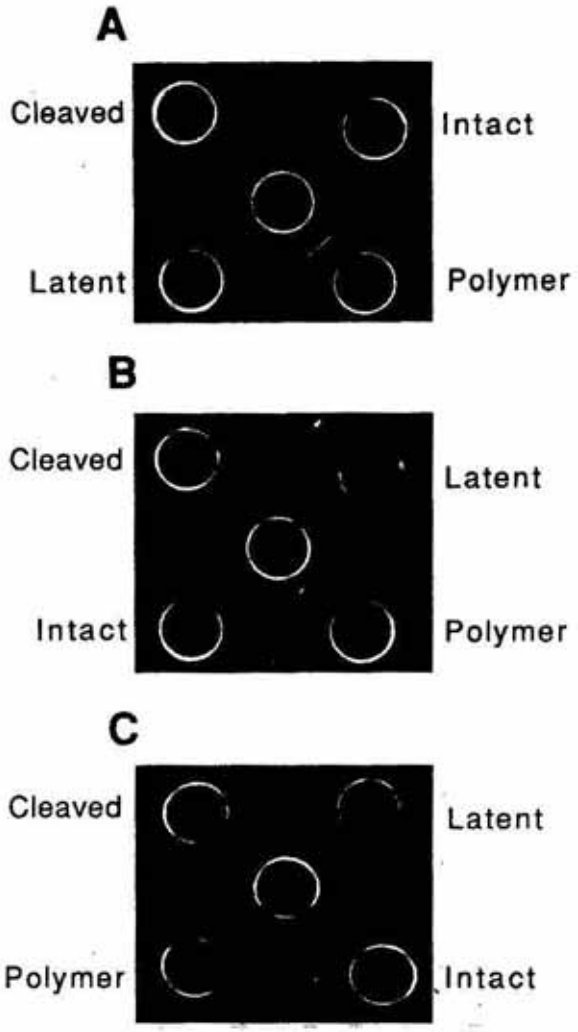

Fig. 2. Immunodiffusion analyses of intact, latent, polymer and cleaved forms of human α_1-PI by means of antiserum against the GuHCl polymerized inhibitor.
A, The whole antiserum against polymerized α_1-PI was used, the antigens concentrations were equal to 1 mg/ml; B, The same antiserum absorbed with intact inhibitor; the antigens concentration applied was equal either to 1 mg/ml or 0.5 mg/ml (C).

polymers of the inhibitor. The immunological cross-reactivity experiments showed that the antiserum against the polymerised inhibitor gave precipitation lines with all the studied forms of α_1-PI (Fig. 2A). Absorption of the serum with intact α_1-PI removed the reactivity against the native inhibitor while weak precipitation arcs with cleaved, latent and polymerised inhibitor forms were still observed at antigens' concentration of 1 mg/ml (Fig. 2B). The precipitation line of cleaved α_1-PI vanished when the antigens' concentration was diminished to 0.5 mg/ml, while the respective lines of the latent and polymerised forms of the in-

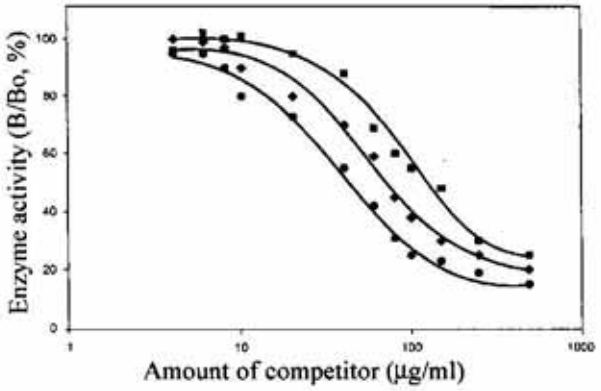

Fig. 3. Competitive inhibition by various forms of α_1-PI for binding antibodies against GuHCl-induced polymers measured with ELISA.
The antiserum used in this experiment was preabsorbed with intact α_1-PI and the GuHCl-polymers' form was used as a competitor. Abscissa: percentage of bound-enzyme activity without (B_o) and with (B) the competitor; ordinate: log concentration of the competitor. (■), Latent form; (◆), α_1-PI polymers and (●) cleaved α_1-PI. The competitor concentrations required for 50% inhibition of antibody binding to antigens were as follows: for latent form — 110 mg/ml, GuHCl-induced polymers — 75 mg/ml and cleaved α_1-PI — 52 mg/ml.

hibitor were still visible (Fig. 2C). The data suggest that the cleaved α_1-PI can have different epitopes or a smaller number of the same group of epitopes compared to the latent and polymerised forms of α_1-PI. For detailed quantitative studies the competitive ELISA was applied. As it is shown in Fig. 3, the inhibition curves for particular antigens were significantly different indicating either non-identity in terms of the recognized epitopes or differences in binding affinity of the same antigenic determinants. Differences in the concentration of the competitor required to give 50% inhibition of the antiserum binding indicate that the antibodies reacted less efficiently with the latent form than with the polymerised and cleaved ones (see legend to Fig. 3). In order to obtain 50% inhibition of antibody binding to the latent antigen, the amount of competitor had to be higher by about 30% compared to that for the polymerised form. On the other hand, for 50% inhibition of binding to the cleaved α_1-PI the amount of the competitor required was lower almost by a half compared to that necessary for blocking of binding to the latent form. This may suggest that the cleaved and polymer forms of α_1-PI shared more of common epitopes than did polymers and the latent forms.

To characterise further the differences between the GuHCl-induced polymer and latent form of α_1-PI, the antiserum against the polymers was quantitatively absorbed with the cleaved inhibitor and used in ELISA experiments. This time either latent or native inhibitor was used as a competitor. As shown in Fig. 4A, antibodies raised against GuHCl-induced polymers reacted very weakly with the determinants present on the native inhibitor.

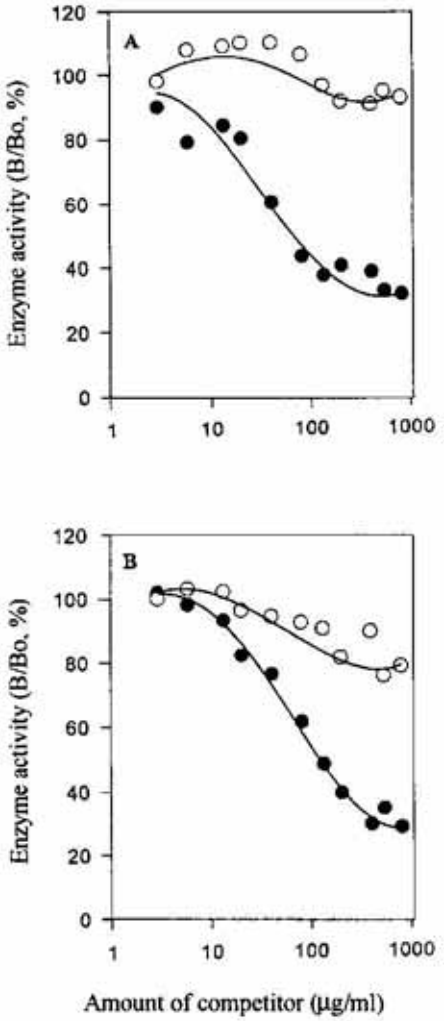

Fig. 4. Competitive inhibition of antiserum binding to GuHCl-induced polymers (A) and latent form (B) of α_1-PI.
The antiserum against GuHCl-induced polymers was quantitatively absorbed with cleaved α_1-PI. The inhibition of antibodies binding was carried out with native (○) and latent (●) forms as competitors. The 50% inhibition coefficient of antibody binding to polymers and latent form as antigens was equal to 80 μg/ml (A) and 50 μg/ml (B), respectively, when the latent form was used as a competitor. The 50% inhibition of binding for native competitor was not estimated because the decrease in enzyme activities was small over the whole competitor concentration range studied.

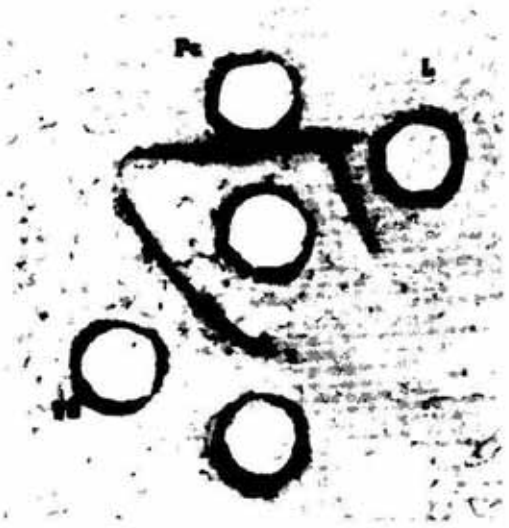

Fig. 5. Immunodiffusion analyses of (L) latent, (V8) cleaved and (Pc) citrate-induced polymer forms of α_1-PI with the use of an antiserum against cleaved α1-PI.

The same measurements were carried out with latent form as an antigen. As can be seen from Fig. 4B, both native and latent competitors eliminated some fraction of the antibodies against GuHCl-induced polymers. Over the whole competitor concentration studied the binding of the antibodies to the latent antigen was decreased by about 20% when the native competitor was applied. On the basis of the data presented in Figs. 3 and 4 we have come to the conclusion that the polymerised form of α_1-PI has a very small number of determinants characteristic of native molecule, and about 40% less of the determinants characteristic of the latent antigen. A comparison of the inhibition curves for native competitor in Fig. 4 A and B suggest that the latent form of inhibitor shares some epitopes with the native molecule.

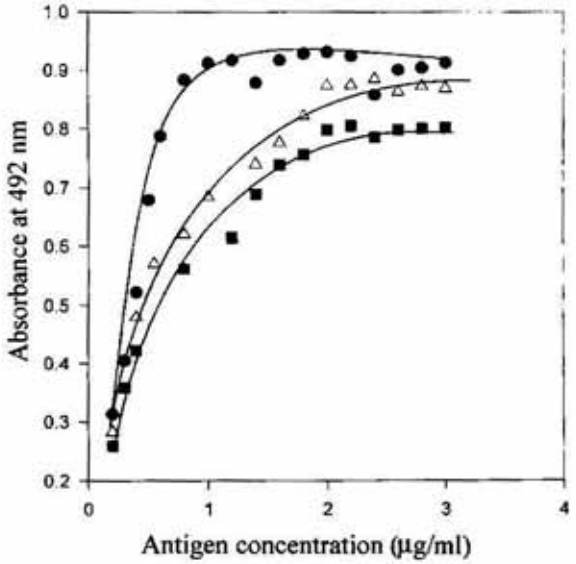

Fig. 6. The curves of (●) cleaved, (Δ) citrate-induced polymers and (■) latent antigens binding to antiserum raised against cleaved α_1-PI.

Our recently published fluorescence stopped-flow measurements of citrate-induced polymers suggest that these polymers contain the latent component in which the RSL is inserted into the A-sheet [13]. To check this conclusion we applied the immunodiffusion and ELISA methods with antibodies raised against the cleaved inhibitor. In our opinion these antibodies should mainly monitor the characteristic structural features related to insertion of the reactive loop into the A-sheet. The data from the immunodiffusion experiment are presented in Fig. 5. As can be seen, the latent form of the inhibitor does indicate partial immunological indentity with the citrate-induced polymers. The quantitative ELISA measurements were applied to confirm it (Fig. 6). The binding curves of the latent and citrate-induced polymers show a similar profile. This can be due to the presence of the same groups of epitopes on these antigens.

DISCUSSION

The results presented show that the GuHCl-induced polymers of α_1-PI are not immunologically identical with the latent form. If the reactive loop insertion into the A-sheet induces new epitopes characteristic of the latent form, it is understandable that some epitopes of the polymer form are hidden and not available to antibodies. It has been suggested that polymerisation of α_1-PI takes place by insertion of a protruded reactive loop of one molecule into the A-sheet of another [6]. This process is accompanied by conformational changes [14]. Our experiments have proved that the GuHCl-induced polymer and latent forms of α_1-PI differ in terms of immunological identity. However, we can state that conformational differences between these forms are due to masking, or exposure of some new epitopes in the polymers' state. If a reactive loop insertion is to be the mechanism of polymerisation, the final structure of a polymer form should resemble either the latent or cleaved α_1-PI, as it is in the

case of a synthetic peptide annealing into the A-sheet [15]. Our data have shown that the structure of α_1-PI polymer form is distinguishable from both those of the latent and cleaved forms of α_1-PI. However, it can be stated that the structural differences between the GuHCl-induced polymer and cleaved forms are significantly smaller than those between the polymer and latent inhibitor. We suppose that in the latent form of α_1-PI the insertion of the reactive loop into the A-sheet is incomplete and therefore its conformation does not fully resemble that of α_1-PI cleaved structure. This conclusion nicely corresponds to the data of Lomas *et al.* [4] who have confirmed partial insertion of RSL into the A-sheet. It may also explain the differences in immunological response between the latent and cleaved forms observed in Fig. 3. On the other hand, the detected differences between polymers and cleaved forms, estimated to amount only to about 30% can suggest that these forms shared common structural features. If such similarities result from the insertion of the RSL into the A-sheet then the polymerisation mechanism throughout the insertions of the RSL will be highly probable.

We have observed a different immunoreactivity of the latent and cleaved forms of α_1-PI with the same antiserum. On the other hand, Björg *et al.* [17] have reported no immunological differences between latent and cleaved ATIII. Since it is believed that conclusions referring to one member of the serpins family are valid for the whole family, our results concerning the differences in immunological properties of the latent and cleaved forms of α_1-PI are rather unexpected. The discrepancy can be explained by the fact that ATIII inserts the reactive loop under mild conditions much more readily and mimics fully the cleaved inhibitor. Using the laser scattering light measurements, we have observed that under mild conditions the aggregation of ATIII runs faster than that of α_1-PI (not shown). From estimation of the inhibition coefficient (Fig. 3), we can judge that our cleaved inhibitor shares about 50% of identical epitopes with the α_1-PI latent form. This conclusion is in agreement with the observation of Carrell's group [2] in terms of structural differences between latent and cleaved ATIII when they were dealing with a partially inserted loop of this protein.

Polymerisation of α_1-PI is restrained in the presence of 0.7 M sodium citrate leading to formation of short polymers [4]. Probably, the mechanism of such polymerisation is based on incorporation of latent species into the polymers' molecule, which terminates chain extention. The data obtained with antiserum against the cleaved inhibitor seem to imply that the citrate-induced polymers contain the latent component (Figs. 5 and 6).

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