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# Interaction of Human Tissue Plasminogen Activator (t-PA) with Pregnancy Zone Protein: A Comparative Study with t-PA-$\alpha_2$-Macroglobulin Interaction[1]

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**Human pregnancy zone protein (PZP) is a major pregnancy-associated plasma protein strongly related to $\alpha_2$-macroglobulin ($\alpha_2$-M). Interactions of tissue plasminogen activator (t-PA) with PZP and $\alpha_2$-M were both investigated *in vitro* and the complexes were analyzed by polyacrylamide gel electrophoresis (PAGE). The results demonstrated that PZP-t-PA complex formation was evident within 1 h of incubation, whereas $\alpha_2$-M-t-PA complexes were formed after 18 h. Conclusions were supported by the following evidence: (i) PZP and $\alpha_2$-M complexes revealed changes of the mobility rate in non-denaturing PAGE, similar to those observed with $\alpha$-Ms-chymotrypsin; (ii) both PZP and $\alpha_2$-M formed complexes of molecular size >360 kDa by SDS-PAGE, in accordance with the covalent binding of t-PA, which was previously reported for other proteinases; and (iii) PZP underwent a specific cleavage of the *bait* region with appearence of fragments of 85-90 kDa as judged by reducing SDS-PAGE. In contrast, the proteolytic attack on $\alpha_2$-M was found to occur more slowly, requiring several hours of incubation with t-PA for generation of an appreciable amount of fragments of 85-90 kDa. The appearance of free SH-groups of $\alpha$-Ms was further investigated by titration with 5,5′-dithiobis(2-nitrobenzoic acid). The maximal level of SH-groups raised was 3.9 mol/mol of PZP and 3.5 mol/mol of $\alpha_2$-M, indicating approximately one SH-group for each 180-kDa subunit. Finally, t-PA activity in PZP-t-PA complex was evaluated by measuring the hydrolysis of the chromogenic substrate Flavigen t-PA. Our results revealed that prolongation of the incubation period of this complex increased t-PA-mediated hydrolysis of Flavigen t-PA until a plateau was reached, approximately between 60 and 120 min. The present study suggests that PZP, by binding to t-PA, may contribute to the control of the activity of proteinases derived from fibrinolytic systems.**

**Key words: fibrinolysis, $\alpha_2$-macroglobulin, pregnancy zone protein, tissue plasminogen activator, trypsin.**

Pregnancy zone protein (PZP) is the most abundant pregnancy-associated plasma protein, exhibiting strong similarities to $\alpha_2$-macroglobulin ($\alpha_2$-M). Both are glycoproteins with strong proteinase-inhibitory activities which are composed of 180-kDa subunits (*1*). While native $\alpha_2$-M exists exclusively in a tetrameric form (720 kDa) composed of a non-covalently associated dimer of disulphide-linked dimers (360 kDa), PZP exists predominantly as disulphide-linked 360-kDa dimers (*2*). These $\alpha$-macroglobulins ($\alpha$-Ms) possess the unique mechanism of covalently binding proteinases without inhibiting their active sites, but they sterically hinder the bound proteinases from being catalytically active against high-molecular-mass substrates (*3*). When $\alpha$-Ms interact with a proteinase, a peptide bond localized within the so-called *bait* region of the inhibitor molecule is cleaved. This event leads to a conformational change in the macroglobulins, which is accompanied by the "trapping" of the proteinase, activation and cleavage of internal $\beta$-cysteinyl-$\gamma$-glutamyl thiol esters, and exposure of receptor recognition sites important for its rapid clearance from the circulation and tissues (*4-6*). The conformationally-altered form of $\alpha_2$-M is structurally more compact and can be readily distinguished from native unaltered forms by non-denaturing PAGE as a band with faster electrophoretic mobility (*7*). In contrast, a slower mobility rate of PZP-proteinase complexes is detectable by non-denaturing PAGE, compared to the mobility rate of native PZP corresponding to its tetrameric form (*2*).

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Abbreviations: $\alpha_2$-M, $\alpha_2$-macroglobulin; $\alpha$-Ms, $\alpha$-macroglobulins; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; PZP, pregnancy zone protein; t-PA, tissue plasminogen activator.

In healthy males and non-pregnant females plasma, PZP is a trace protein (<10 mg/liter) which may reach maximum levels of approximately 300 mg/liter during late pregnancy (*8-10*), while in the same situation plasma levels of $\alpha_2$-M remain constant (*11*). The physiological significance of proteinase inhibition by PZP is largely unknown and remains to be elucidated, but its temporal appearance during gestation and subsequent disappearance after parturition suggest that it could play a specific role in the control of pregnancy-associated proteolytic processes (*2*). Interestingly, during pregnancy, the fibrinolytic activity has been shown to be reduced (*12-16*). This event may be related to the levels of plasma proteinase inhibitors interacting with serine proteinases of the fibrinolytic system (*17*). In this context, tissue plasminogen activator (t-PA) is the principal plasmatic fibrinolytic activator (*18*), and thus, its inhibition could represent an explanation for downregulation of the fibrinolytic activity.

In the present work, we studied the interactions of t-PA with PZP and $\alpha_2$-M, and discussed the behaviour of their respective complexes on PAGE under different conditions. Furthermore, we report the appearance of free SH-groups of $\alpha$-Ms and the functional enzymatic activity of t-PA in PZP-t-PA complexes.

## EXPERIMENTAL PROCEDURES

*Purification of $\alpha_2$-M and PZP*—Human $\alpha_2$-M and PZP were isolated from pooled late pregnancy plasma as described by Chiabrando *et al.* (*19*) and stored at 0°C in 0.1 M sodium-phosphate pH 8.0 until use. Both were more than 95% active at the time of the assay, as determined by titration of SH-groups appearing from thiol ester cleavage following addition of excess chymotrypsin. The concentration of $\alpha_2$-M and PZP were determined by measuring their optical density at 280 nm, using an extinction coefficient ($E^{1\%}$) of 8.9 for $\alpha_2$-M (*20*) and 8.2 for PZP (*2*).

*Enzymes, Antibody and Chemicals*—The peptide substrate Flavigen™ t-PA ($CH_3SO_2$-D-HHT-Gly-Arg-pNA AcOH) and human two-chain tissue plasminogen activator (t-PA) were purchased from Biopool AB, Umeå, Sweden. t-PA was more than 95% two chains (70 kDa) and had a fibrinolytic activity of 780,000 IU per mg. The activity of t-PA was tested by the method of Rånby *et al.* (*21*). Goat anti-human PZP was obtained in our laboratory as described (*22*). Bovine α-chymotrypsin, bovine trypsin, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma Chemical (USA). All other chemicals were analytical grade.

*Enzyme-Inhibitor Complexes Assays*—Native $\alpha_2$-M (0.2 μM) was incubated at room temperature (22°C) in 0.1 M sodium phosphate, pH 8.0, with 0-0.4 μM t-PA in a total volume of 2.0 ml. In the case of PZP, reaction mixtures containing 0.25 μM PZP (720 kDa) were incubated with 0-0.5 μM t-PA under the same conditions as $\alpha_2$-M. Aliquots of mixtures were then subjected to non-denaturing PAGE analyses. Samples for non-reducing and reducing SDS-PAGE were prepared from incubation mixtures containing $\alpha_2$-M (0.2 μM) and PZP (0.25 μM) with 0.4 and 0.25 μM t-PA, respectively, under the conditions described above. As control, chymotrypsin-treated α-Ms samples were incubated at a molar ratio of 1:1. At different times, all the reactions were stopped by addition of PMSF at a final concentration of 1.0 mM and incubation for at least 10 min.

*PAGE*—Non-denaturing PAGE was performed on a 5% gel as described by Van Leuven *et al.* (*23*). A discontinuous buffer system was used. Samples were prepared in reservoir buffer (0.041 M Tris, 0.04 M sodium borate, pH 8.6) containing 10% glycerol and separated by electrophoresis for 3 h at constant 150 V. SDS-PAGE was carried out as described by Laemmli (*24*). Samples were prepared under both reducing and non-reducing conditions; *i.e.*, diluted with sample buffer either with or without dithiothreitol and incubated for 45 min at 37°C. Aliquots of 12-35 μl containing 2-5 μg protein were loaded on the gels. Electrophoresis were performed using a Mini-Protean II electrophoretic cell (Bio Rad, Richmond, CA), and proteins were detected by silver staining (*25*).

*Thiol Titration*—The exposure of SH-groups in PZP and $\alpha_2$-M reactions with enzymes was followed by measuring changes in the absorbance at 412 nm using a Shimadzu UV-1601 PC in the presence of a large excess of DTNB, as was essentially described for $\alpha_2$-M-proteinase reactions (*26*). Mols of SH-groups generated per mol of α-Ms were calculated by using a molar absorptivity of 13,600 $M^{-1} \cdot cm^{-1}$ (*27*).

*Amidolytic Activity of t-PA in PZP-t-PA Complexes*—To ascertain the rate of complex formation as a function of bound t-PA, we developed a PZP-proteinase binding assay. These assays were performed by coating microtiter plates (96 wells) with 10 μg/ml goat IgG anti-human PZP in 0.1 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.6, for 3 h at 37°C. Plates were washed five times with 0.01 M phosphate buffer, pH 7.4, with 0.123 M sodium chloride containing 0.5 g/liter Tween 80 (PBS-$T_{80}$), then air-dried and stored at 4°C (*22*). Reaction mixtures of PZP and t-PA at molar ratios of 1:1 and 1:2 were incubated for 0, 30, 60, 120, 180, and 240 min at 22°C. Aliquots of the reaction mixtures were diluted 1:50 with PBS-$T_{80}$ to obtain a final concentration of 5 μg/ml of PZP, and 100-μl portions of these diluted samples were added to each well and incubated for 3 h at 37°C. Plates were then washed, and t-PA activity was measured after addition of 100 μl of the chromogenic substrate Flavigen t-PA following the manufacturer's recommended protocol. After 8 h of incubation at 37°C, the absorbance at 405 nm was recorded using a Bio Rad microplate reader. A calibration curve was run in each microplate by using several dilutions of purified t-PA (from 0.03 to 1.0 IU/ml), and the activity of t-PA in PZP-t-PA complexes was measured.

## RESULTS

*Electrophoretic Analysis of the Interactions between t-PA and α-Ms*—To establish the possible interactions of t-PA with PZP and $\alpha_2$-M, reaction mixtures of these α-Ms and proteinases, incubated at several molar ratios for different periods were studied by non-denaturing PAGE. As Fig. 1 clearly shows, PZP changed its electrophoretic mobility when incubated for 1 h with t-PA at a molar ratio of 0.5-2.0, in comparison to its native form. This mobility change was a result of the conversion of dimeric into tetrameric PZP, which was in agreement with that found for PZP-chymotrypsin complex. Since chymotrypsin is the best characterized proteinase which forms complexes with PZP (*28*), it was used as a control for these electrophoretic

assays. In the case of $\alpha_2$-M (Fig. 2), only when it was incubated with t-PA for as long as 18 h at a molar ratio of 1:2 was a significative conversion from the slow to the fast form observed, with similar mobility to that found for $\alpha_2$-M-chymotrypsin complex.

To demonstrate the covalent binding between PZP and t-PA, we analyzed by non-reducing SDS-PAGE the interaction between PZP and t-PA (molar ratio 1:1) incubated for 1 h at room temperature. Under these conditions, Fig. 3A shows that PZP covalently bound t-PA by forming stable complexes of very high apparent molecular size (>360 kDa) as compared to native PZP. This effect was clearly observed when $\alpha_2$-M was incubated with t-PA (molar ratio 1:2) for 18 h (Fig. 3B). Furthermore, when $\alpha$-Ms-t-PA complexes were analyzed under reducing SDS-PAGE, the appearance of characteristic fragments of 85-90 kDa, indicative of proteolytic cleavage of the *bait* region, were clearly visualized. On the other hand, Fig. 4 shows that PZP incubated with t-PA for 1 h at a molar ratio 1:1 exhibited a complete proteolytic cleavage of the *bait* region, evidenced by disappearance of the 180-kDa subunit and concomitant detection of fragments of 85-90 kDa. In contrast, $\alpha_2$-M was found to be cleaved by t-PA only after long-term incubation, requiring several hours to obtain an appreciable yield of cleavage products (Fig. 4).

*Thiol Titration*—When PZP was incubated with a two-fold molar excess of t-PA, the appearance of free SH-groups was further investigated by DTNB titration in a time-course study (Fig. 5). For comparison, the release of SH-groups induced by incubating trypsin with PZP is also shown. As is clearly evidenced, PZP reacted with t-PA and trypsin at comparable rates, releasing the maximal level of SH-groups within 60 min, while reaction of PZP with chymotrypsin was found to be faster (<5 min) (Fig. 5, insert). Table I shows that the level of SH-groups appearing in PZP after incubation or preincubation with chymotryp-

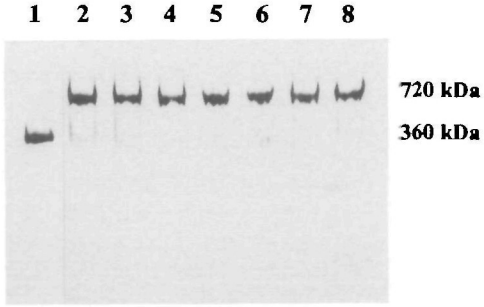


Fig. 1. **Analysis by non-denaturing PAGE of the reaction between t-PA and PZP.** At different time periods, aliquots containing 2-5 $\mu$g of proteins were taken as described in "EXPERIMENTAL PROCEDURES" and analyzed on a 5% acrylamide slab gel. Lane 1: native PZP; lane 2: PZP incubated with chymotrypsin for 5 min at a ratio of 1:1; lanes 3-5: PZP incubated with t-PA for 1 h at molar ratios of 1: 0.5; 1:1, and 1:2. Lanes 6-8: PZP incubated with t-PA for 2 h at the same molar ratios as described above. Proteins were detected by silver staining.

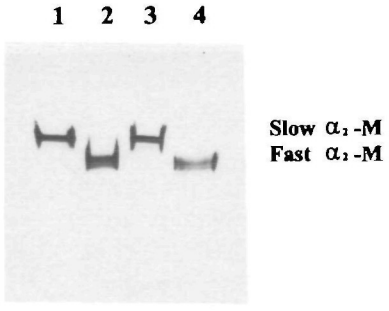


Fig. 2. **Analysis by non-denaturing PAGE of the reaction between t-PA and $\alpha_2$-M.** At different time periods, aliquots containing 2-5 $\mu$g of proteins were taken as described in "EXPERIMENTAL PROCEDURES" and analyzed on a 5% acrylamide slab gel. Lane 1: native $\alpha_2$-M; lane 2: $\alpha_2$-M incubated with chymotrypsin for 5 min at a molar ratio of 1:1; lane 3: $\alpha_2$-M incubated with t-PA for 1 h at a molar ratio of 1:2; lane 4: $\alpha_2$-M incubated with t-PA for 18 h at a molar ratio of 1:2. Proteins were detected by silver staining.

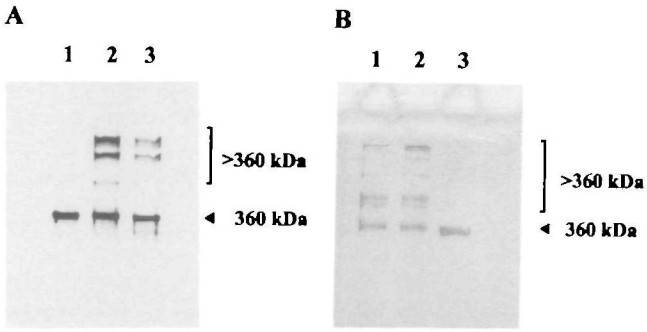


Fig. 3. **Analysis by SDS-PAGE of the reaction between t-PA and $\alpha_2$-M or PZP.** Aliquots were collected at different times as described in "EXPERIMENTAL PROCEDURES" and analyzed on a 4% acrylamide slab gel. (A) Lane 1: native PZP (360 kDa); lane 2: PZP incubated with chymotrypsin for 5 min at a molar ratio of 1:1; lane 3: PZP incubated with t-PA for 1 h at a molar ratio of 1:1. (B) Lane 1: $\alpha_2$-M incubated with t-PA for 18 h at a molar ratio of 1:2; lane 2: $\alpha_2$-M incubated with chymotrypsin for 5 min at a molar ratio of 1:1; lane 3: native $\alpha_2$-M. Prior to electrophoresis, samples were treated with 2% SDS for 45 min at room temperature, and approximately 2-5 $\mu$g of incubation mixtures were applied to the gels.

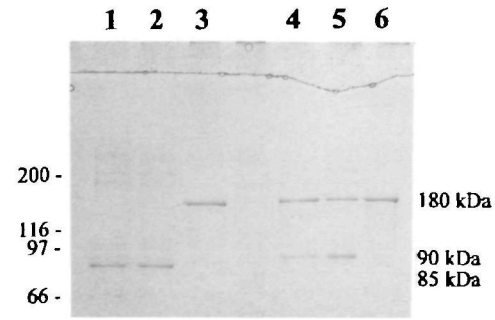


Fig. 4. **Analysis by SDS-PAGE under reducing conditions (33 mM DTT) of the reaction between t-PA and chymotrypsin with $\alpha_2$-M or PZP.** Aliquots were collected at different times as described in "EXPERIMENTAL PROCEDURES" and analyzed on a 3.5-10% linear-gradient acrylamide slab gel. Lane 1: $\alpha_2$-M incubated with t-PA for 18 h at a molar ratio of 1:2; lane 2: $\alpha_2$-M incubated with chymotrypsin for 5 min at a molar ratio of 1:1; lane 3: native $\alpha_2$-M; lane 4: PZP incubated with t-PA for 1 h at a molar ratio of 1:1; lane 5: PZP incubated with chymotrypsin for 5 min at a molar ratio of 1:1; lane 6: native PZP. Proteins were detected by silver staining. Positions of electrophoretic markers are indicated on the gel; from top to bottom: myosin (200 kDa), $\beta$-galactosidase (116 kDa), phosphorylase B (97 kDa), serum albumin (66 kDa).

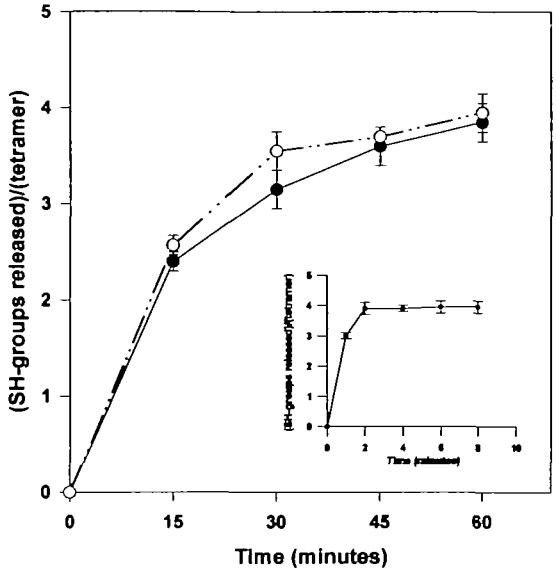


Fig. 5. **Release of DTNB-titrable SH-groups in PZP as a result of incubation with t-PA (●) and trypsin (○).** Concentration of PZP was 2.4 μM in 0.1 M sodium phosphate, pH 8.0. Following addition of DTNB (2 mM final concentration), PZP was mixed with twofold molar excess of proteinase and the change in absorbance at 412 nm was recorded until no further significant changes occurred (after 60 min), corresponding to approximately 3.9-4.0 mol SH-groups per mol PZP tetramer. Each point represents the mean ± SE of three replicates. Insert: Release of DTNB-titrable SH-groups in PZP as a result of incubation with chymotrypsin (up to 8 min) under the conditions described above.

TABLE I. **Thiol titration of proteinase-treated α-macroglobulins.**

| α-Macroglobulins (PZP[a] and $\alpha_2$-M[b]) | SH released/tetramer (mol/mol) |
|---|---|
| Native PZP | 0 |
| PZP-chymotrypsin | 4.0 |
| PZP-trypsin | 4.0 |
| PZP-t-PA | 3.9 |
| Native $\alpha_2$-M | 0 |
| $\alpha_2$-M-chymotrypsin | 4.0 |
| $\alpha_2$-M-t-PA | 3.5 |

[a]Pregnancy zone protein (2.4 μM) was incubated or preincubated for different periods with chymotrypsin, trypsin, or t-PA, simultaneously with or prior to DTNB addition under the conditions described in "EXPERIMENTAL PROCEDURES." [b]$\alpha_2$-M (2.0 μM) was incubated for 5 min with chymotrypsin or preincubated for 18 h with t-PA prior to DTNB addition.

sin, trypsin, or t-PA was 3.9-4.0 mol per mol of tetramer, indicating that each of these four subunits contributes one SH-group. A similar release of SH groups was observed when PZP was preincubated with t-PA or trypsin for 60 min and then titrated with DTNB solution (Table I).

In contrast, when $\alpha_2$-M was incubated with t-PA at a molar ratio of 1:2, no significative changes in the absorbance were observed up to 180 min, while chymotrypsin reacted with $\alpha_2$-M at a similar rate to that observed with PZP (<5 min), showing that the level of SH-groups released was 4.0 mol per mol of tetramer (Table I). However, when $\alpha_2$-M and t-PA were preincubated for 18 h and the SH-groups were further titrated, $\alpha_2$-M showed 3.5 mol of SH-groups per mol of tetramer (Table I).

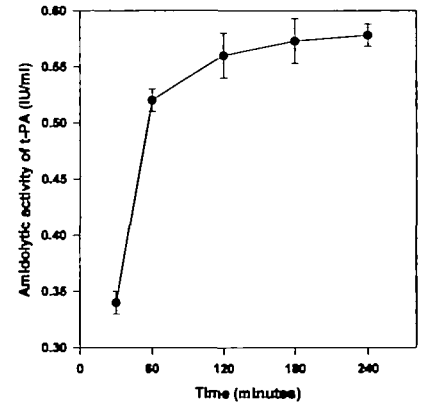


Fig. 6. **Amidolytic activity of t-PA in PZP-t-PA complexes.** Tissue plasminogen activator was exposed to PZP at a molar ratio of 1:1 for 0, 30, 60, 120, 180, and 240 min, and the amidolytic activity was determined by measuring the extent of hydrolysis of the chromogenic substrate Flavigen™ t-PA. The present result is representative of three independent experiments.

*Amidolytic Activity of t-PA in PZP-t-PA Complexes*—To ascertain the rate of complex formation as a function of bound t-PA, the proteinase was incubated with PZP at molar ratios of 1:1 and 1:2 for 0, 30, 60, 120, 180, and 240 min, and the amidolytic activity was measured by using the chromogenic substrate Flavigen t-PA. As evidenced in Fig. 6, the amidolytic activity of PZP-t-PA increased until a plateau was reached within 60 to 120 min of incubation. This finding could indicate a saturation of the amount of bound t-PA with PZP within 2 h of incubation, in accordance with our previous results. Similar results were obtained by using a molar ratio of 1:2 (data not shown).

## DISCUSSION

It is widely reported and accepted that $\alpha_2$-M is one of the major proteinase inhibitors of the blood, which is capable of binding and clearing most proteinases from the circulation. This broad specificity suggests that it represents a general defense against proteolytic attack (*29*). In contrast, the physiological role of proteinase inhibition by PZP is still unknown and remains to be elucidated. Pregnancy is the classic state in which PZP plasma levels increase significantly. Strikingly, the fibrinolytic activity is decreased during this period. In the present work, we demonstrate by PAGE analysis that PZP interacts with t-PA within 1 h of incubation at room temperature, whereas $\alpha_2$-M reacts over a longer period of up to 18 h. Formation of α-macroglobulin-t-PA complexes was evidenced by a number of observations, as follows: (a) PZP and $\alpha_2$-M showed changes in their mobility rates in non-denaturing PAGE comparable to those observed with chymotrypsin (*2, 3, 28*); (b) both PZP and $\alpha_2$-M, formed complexes of molecular size >360 kDa in agreement with their covalent binding to t-PA, as reported for other proteinases (*30-32*); and (c) PZP underwent a specific cleavage of the *bait* region with rapid appearance of fragments of 85-90 kDa as judged by reducing SDS-PAGE. In contrast, this proteolytic attack on $\alpha_2$-M as found to be slow, requiring several hours of incubation with t-PA for generation of an appreciable amount of fragments of 85-90 kDa (*2, 28*).

It is generally known that PZP and $\alpha_2$-M have different proteinase-binding abilities (*33*). While each human $\alpha_2$-M required one or two molecules of proteinases for generating a mobility change on native PAGE, the formation of tetrameric PZP by native PAGE demonstrated that a 1:1 molar ratio complex of proteinase to PZP tetramer is most likely to appear (*2, 28*). In our approach, one molecule of t-PA for each of PZP was enough to induce the same modification, according to previous results reported for other proteinases (*2*).

Furthermore, incubation of PZP with t-PA, trypsin, or chymotrypsin resulted in the appearance of SH-groups, similar to that found for $\alpha_2$-M (*26*). However, the release rate of SH-groups differed greatly. For both PZP and $\alpha_2$-M, incubation with chymotrypsin resulted in fast thiol ester cleavage, whereas for t-PA and trypsin, thiol ester cleavage in PZP was relatively slow, requiring incubation of 60 min for the appearance of approximately four moles of thiol groups per mol of tetramer (Table I). In contrast, $\alpha_2$-M with t-PA required a longer reaction period of more than 18 h in order to produce an amount of 3.5 SH-groups mol per mol of $\alpha_2$-M. The different reaction times observed herein for $\alpha$-Ms are in agreement with those reported in previous studies (*34, 35*), which indicate that the overall rate of complex formation with different proteinases leading to thiol ester cleavage may differ by several orders of magnitude. This could be explained by taking into consideration the fact that sequences of the *bait* region of human $\alpha$-Ms are dissimilar and exhibit different lengths (*1, 36, 37*). The sites of proteolytic cleavage have been extensively mapped (*1, 38, 39*) and generally conform to the primary substrate specificity of the proteinase in question. Several proteinases including trypsin, chymotrypsin, and elastase react rapidly with $\alpha_2$-M, while restrictive proteinases such as thrombin and plasmin react slowly (*1, 34, 40*). Evidently, each *bait* region contains one or more peptide bonds which can be cleaved by a wide variety of proteinases. However, due to the diversity of the *bait* region sequences, the $P_4$-$P_4'$ residues at the site(s) of cleavage differ greatly among $\alpha$-Ms. The sequence of the PZP *bait* region contains no lysyl residues and only one arginyl residue (in the sequence Arg-Pro) at which fast cleavage by trypsin-like enzymes could take place (*39*). Hence, this region is not expected to be cleaved easily by those proteinases. If we assume that t-PA is a trypsin-like proteinase, it must be inferred that it reacts with PZP in a similar way to trypsin. Even when there is no specific recognition site for trypsin in the *bait* region, in our studies t-PA was able to cleave PZP within 60 min. Why PZP and $\alpha_2$-M react differently with t-PA remains to be elucidated. One possibility is that another recognition site in the *bait* region is present in the PZP molecule. Experimental approaches are now being designed to address this issue. Moreover, we demonstrated by using the chromogenic substrate Flavigen t-PA that PZP binds proteinases without inhibiting their active site, maintaining intact their ability to interact with low molecular weight substrates.

Early studies showed that $\alpha_2$-M plays a key role in the regulation of both coagulation and fibrinolysis (*32, 41-43*). In this context, Arbelaez *et al.* (*44*) reported that PZP exerts inhibition on human tissue kallikrein, but not on other proteinases. Interestingly, in our system we succeeded in inducing inhibition of t-PA, thus widening their observations. Furthermore, disagreement exists among previous reports as to whether $\alpha_2$-M can exert an inhibitory effect on t-PA. In this sense, whereas Wiman *et al.* (*45*) showed by chromatographic and enzymatic analysis that t-PA binds to $\alpha_2$-M after 30 min, Arbelaez *et al.* (*44*) obtained by PAGE analysis the same results only after several hours of incubation. These discrepancies could be explained by the different experimental approaches used. However, in our case, both PZP and $\alpha_2$-M were able to interact with t-PA, although at different times of incubation.

Over the past few years, considerable advances have been made in the understanding of the process of fibrinolysis, and a complex relationship exists between plasminogen activators and inhibitors during pregnancy. Moreover, considering the PAI-1 variations during normal pregnancy, one should expect that other proteinase inhibitors also contribute to the overall control of the fibrinolytic activity. It has been reported that other plasma serine proteinase inhibitors (*e.g.*, $\alpha_2$-antiplasmin, $\alpha_2$-macroglobulin, $\alpha_1$-antitrypsin) interact with plasminogen activators. In this sense, as PZP plasma levels have been shown to be increased during pregnancy and conversely fibrinolytic activity has been shown to be reduced in this physiological state, the findings presented herein suggested that PZP could contribute *in vivo* to the control of the activity of proteinases such as t-PA.

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