unknown, it is still possible to calculate these values from the curve $R_{sat} = f(pH)$ (relationship 4), as the curve is symmetrical with a maximum value at pH = $(pK_1 + pK_2)/2$. From this pH value and the corresponding R_{sat} value, pK_1 and pK_2 can be established.

REFERENCES

Bianco, P., & Haladjian, J. (1981) *Electrochim. Acta 26*, 1001–1004.

Bruschi, M. (1981) *Biochim. Biophys. Acta 671*, 219–226.

Bruschi, M., Hatchikian, E. C., Golovleva, L. A., & Le Gall, J. (1977) *J. Bacteriol. 129*, 30–38.

Bruschi, M., Loutfi, M., Bianco, P., & Haladjian, J. (1984) *Biochem. Biophys. Res. Commun. 120*, 384–389.

Bruschi, M. H., Guerlesquin, F. A., Bovier-Lapierre, G. E., Bonicel, J. J., & Couchoud, P. M. (1985) *J. Biol. Chem. 260*, 8292–8296.

Capeillere-Blandin, C., Guerlesquin, F., & Bruschi, M. (1986) *Biochim. Biophys. Acta 848*, 279–293.

Chothia, C., & Janin, J. (1975) *Nature (London) 256*, 705–708.

Coassolo, P., Sarrazin, M., & Sari, J. C. (1980) *Anal. Biochem. 104*, 37–43.

Dickerson, J. L., Kornuc, J. J., & Rees, D. C. (1985) *J. Biol. Chem. 260*, 5175–5178.

Eley, G. G. S., & Moore, G. R. (1983) *Biochem. J. 215*, 11–21.

Gayda, J. P., Bertrand, P., More, C., Guerlesquin, F., & Bruschi, M. (1985) *Biochim. Biophys. Acta 828*, 862–867.

Guerlesquin, F., Bruschi, M., Bovier-Lapierre, G., & Fauque, G. (1980) *Biochim. Biophys. Acta 626*, 127–135.

Guerlesquin, F., Moura, J. J. G., & Cammack, R. (1982) *Biochim. Biophys. Acta 679*, 422–427.

Guerlesquin, F., Bruschi, M., Astier, J. P., & Frey, M. (1983) *J. Mol. Biol. 168*, 203–205.

Guerlesquin, F., Bruschi, M., & Wuthrich, K. (1985a) *Biochim. Biophys. Acta 830*, 296–303.

Guerlesquin, F., Noailly, M., & Bruschi, M. (1985b) *Biochem. Biophys. Res. Commun. 130*, 1102–1108.

Haser, R., Pierrot, M., Frey, M., Payan, F., Astier, J. P., Bruschi, M., & Le Gall, J. (1979) *Nature (London) 282*, 806–810.

Hazzard, J. T., & Tollin, G. (1985) *Biochem. Biophys. Res. Commun. 130*, 1281–1286.

Mathews, S. (1985) *Prog. Biophys. Mol. Biol. 45*, 1–56.

Mauk, M. R., Reid, L. S., & Mauk, A. G. (1982) *Biochemistry 21*, 1843–1846.

Perutz, M. F., Muirhead, H., Mazzarella, L., Grawther, R. A., Greer, J., & Kilmartin, J. V. (1969) *Nature (London) 222*, 1240–1243.

Pierrot, M., Haser, R., Frey, M., Payan, F., & Astier, J. P. (1982) *J. Biol. Chem. 257*, 14341–14348.

Poulos, R. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 10322–10330.

Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096–3102.

Salemme, F. R. (1976) *J. Mol. Biol. 102*, 563–568.

Sari, J. C., & Belaich, J. P. (1973) *J. Am. Chem. Soc. 95*, 7491–7496.

Sari, J. C., Ragot, M., & Belaich, J. P. (1973) *Biochim. Biophys. Acta 305*, 1–10.

Simondsen, R. P., Weber, P. C., Salemme, F. R., & Tollin, G. (1982) *Biochemistry 21*, 6366–6375.

Waldmeyer, B., & Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5184–5190.

Purification and Characterization of a Tissue Plasminogen Activator–Inhibitor Complex from Human Umbilical Vein Endothelial Cell Conditioned Medium

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ABSTRACT: Tissue plasminogen activator–inhibitor complexes were purified from the conditioned medium of human umbilical vein endothelial cells by affinity chromatography followed by gel filtration. It was found that a single complex was isolated which can exist in two distinct interconvertible conformations. These may be separated by electrophoresis into a form with a 105 000 apparent molecular weight and a form with an 88 000 apparent molecular weight. The particular conformation which predominates may be altered by changing the pH at which preparations are incubated or by including dithiothreitol in incubation buffers. Plasminogen activator enzymatic activity may be partially recovered from purified complexes by incubation in the presence of fibrin. Incubation in 1.5 M NH_4OH results in the dissociation of the complex into two major polypeptides of 67 and 40 kilodaltons (kDa). The 40-kDa protein was isolated by gel filtration high-pressure liquid chromatography. N-Terminal amino acid analysis of this protein revealed three distinct sequences. Two of these were nearly identical and matched the N-terminal sequence recently reported for the native plasminogen activator inhibitor from endothelial cells. The third sequence exactly matched an internal portion of the same protein. The results suggest that the internal sequence is located at the site where the inhibitor is cleaved by tissue plasminogen activator.

During the last few years, evidence has accumulated which indicates that there are specific inhibitors which modulate the activity of the plasminogen activator serine proteases in vivo (Ogston & Walker, 1980; Korninger et al., 1985; Wiman et al., 1984a; Kruithof et al., 1984; Verheijen et al., 1984). Deviations from normal plasma levels of plasminogen activator

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inhibitor have been associated with cardiovascular diseases (Brommer et al., 1984; Hamsten et al., 1985; Bergsdorf et al., 1983; Okamura et al., 1984), high-risk pregnancies (Wiman et al., 1984b), hemostatic disturbances (Chmielewska et al., 1983), and severe viral infections (Colucci et al., 1985; Emeis, 1985; Juhan-Vague et al., 1984). A variety of immunological and enzymatic evidence suggests that the major inhibitor of tissue plasminogen activator (tPA)[1] activity in plasma is synthesized and secreted by the endothelial cells which line the walls of blood vessels (Erickson et al., 1985; Sprengers et al., 1985). This inhibitor has been given the designation PAI-1, and the nucleotide sequence for its cDNA has been reported by several laboratories (Andreasen et al., 1986; Pannekoek et al., 1986; Ny et al., 1986). Although the native inhibitor protein from a variety of cells has been extensively studied, the properties of the tPA–inhibitor complex remain largely unknown. This is particularly true of complexes in which both the tPA and the inhibitor are of endothelial cell origin. Since greater than 90% of the tPA in human plasma is found as a complex and since both the inhibitor and tPA appear to originate in the endothelium (Binder et al., 1979; Collen, 1980), such studies are clearly of importance. In order to further our knowledge in this area, we have been studying the tPA–inhibitor complexes produced by human umbilical vein endothelial cells.

In this report, we show that HUE cell conditioned medium contains only one major tPA–inhibitor complex and that this may be purified by using a combination of affinity chromatography and gel filtration. The tPA in the purified complex exhibits no detectable activity in the absence of fibrin; however, it appears that a portion of its enzymatic activity can be recovered when fibrin is present. The tPA–inhibitor complex can interconvert between two distinct conformations with apparent molecular weights of 105 000 and 88 000 and may be dissociated into its component polypeptides by incubation in 1.5 M NH_4OH. Once dissociated, the inhibitor polypeptide appears as a single-chain protein with a molecular weight of about 40 000. Amino acid sequence data suggest that the inhibitor protein derived from the dissociated complexes has been cleaved at an Arg–Met bond.

Materials and Methods

Materials. PAM-2–Sepharose (a packing consisting of anti-tPA monoclonal antibody bound to Sepharose) and Bowes melanoma tPA were purchased from American Diagnostica Inc. (Greenwich, CT). The reagents and protein standards used for electrophoresis were from Bio-Rad. All other chemicals were purchased from either Sigma or Fisher and were reagent grade or a superior quality.

Culturing of HUE Cells. HUE cells were established in culture by using standard published procedures (Gimbrone, 1976; Jaffe, 1980; Knedler & Ham, 1983). Growth took place at 37 °C in cell factories purchased from Nunc (4000 Roskilde, Denmark) in an atmosphere containing 5–7% carbon dioxide. Direct immunofluorescent antibody staining with anti-human factor VIII antibody was used to confirm that cells were of endothelial origin (Jaffe, 1980). Cells were given fresh medium (medium 199 containing 10% fetal bovine serum) 3 times a week and were subcultured at a 1 to 3 split ratio. After having reached confluency, the serum-containing medium was replaced with serum-free medium, and cells were incubated at 37 °C for an additional 24 h. The conditioned medium (CM) was then decanted off the cells and centrifuged at low speed to remove any debris that might be present. CM was stored frozen at −20 °C and served as the starting material for our purifications.

tPA Antigenic Activity. The amount of tPA antigen present in various preparations was determined by using the Imubind tPA ELISA assay kit marketed by ADI. This is a double-antibody sandwich-type assay utilizing polyclonal antibodies against tPA. Experiments in which tPA was titrated into inhibitor-containing HUE-CM indicated that the antibodies reacted nearly as well with tPA–inhibitor complexes as they did with free tPA.

tPA Enzymatic Activity. A tPA enzymatic assay from ADI was adapted for use in these studies. The assay is based on the parabolic rate assay described by Ranby et al. and Wiman (Ranby et al., 1982; Wiman, 1983). tPA standards and samples are incubated in buffer (0.02 M sodium phosphate, pH 6.8, 0.15 M NaCl, and 0.05% Tween 20) in the presence of fibrin, plasminogen, and a chromogenic substrate of plasmin. Each standard and sample is run in the presence of normal goat IgG as well as in the presence of goat anti-tPA IgG. Incubation proceeds at 37 °C for a period of 2 or more h, and absorbance readings are taken at regular intervals. When standards are run in the presence of normal IgG, the parabolic curve obtained for each concentration of tPA represents the total proteolytic activity present in that standard. When the same standards are assayed in the presence of anti-tPA IgG, the amount of non-tPA protease activity is measured. In order to determine tPA-specific protease activity, non-tPA protease activity is subtracted from total protease activity. The amount of tPA enzymatic activity present in a particular sample can be measured by comparing the graph obtained for that sample with the graphs produced by the tPA standards.

Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Gels were silver stained by using the procedure of Morrissey (1981).

HPLC Fractionation. High-performance liquid chromatography (HPLC) was performed on a Hewlett-Packard 1090A liquid chromatograph using size-exclusion GF-250 columns (Du Pont, Wilmington, DE). Size-exclusion HPLC was used to purify both the tPA–inhibitor complex and the tPA inhibitor.

Amino Acid Sequencing. Automated Edman degradation chemistry was used to determine the NH_2-terminal protein sequence. An Applied Biosystems Inc. Model 470A gas-phase sequencer (Foster City, CA) was employed for the degradations (Hunkapiller et al., 1983). The respective PTH-amino acid derivatives were identified by reversed-phase HPLC using an Applied Biosystems Inc. Model 120A PTH analyzer fitted with a Brownless 2.1-mm i.d. PTH–C18 column.

Total Protein Determinations. Total protein in samples was estimated according the procedure of groves et al. in which absorbance readings at 236 nm are subtracted from readings at 224 nm (Groves et al., 1968). Bovine serum albumin was used as the standard.

Results

Purification of tPA–Inhibitor Complex by Affinity Chromatography and Gel Filtration. On the basis of ELISA assays, it was found that the amount of tPA antigenic activity present in different preparations of HUE cell CM varies between 20 and 80 ng/mL. In every instance, the amount of tPA enzymatic activity was below the detection limit of our

[1] Abbreviations: PA, plasminogen activator; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; HUE, human umbilical vein endothelial; ADI, American Diagnostica Inc.; CM, conditioned medium; IgG, immunoglobulin G; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; PBS, phosphate-buffered saline; BAE, bovine aortic endothelial; UK, urokinase; kDa, kilodalton(s); SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

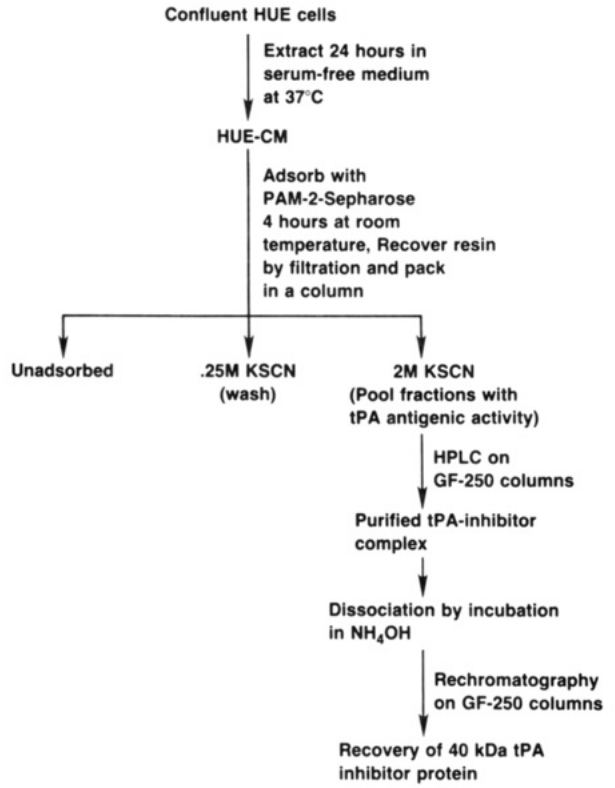

FIGURE 1: Purification procedure for the isolation of tPA–inhibitor complexes and for the isolation of the 40-kDa inhibitor protein.

enzyme assay (0.5 ng/mL), and it was found that all of the tPA antigenic activity eluted from gel filtration columns at a higher molecular weight than would be expected for free tPA. These findings suggest that nearly all of the tPA is present in the form of tPA–inhibitor complexes. These complexes were purified by using the procedure depicted in Figure 1. The first step in the purification involves incubating conditioned medium with PAM-2–Sepharose at a ratio of approximately 2.2 mL of packed resin per liter of CM. Incubation proceeded for 4–5 h at room temperature with constant mixing. The PAM-2–Sepharose was recovered by filtering preparations through coarse fritted glass funnels. It was then washed with PBS–Tween (0.02 M sodium phosphate, pH 7.4, 0.15 M NaCl, and 0.01% Tween 80) and packed in an appropriately sized column. The column was washed with a minimum of 6 column volumes of PBS–Tween containing 0.25 M KSCN and was then eluted with PBS–Tween containing 2.0 M KSCN. Fractions were collected and scanned for tPA antigenic activity using the ELISA assay. Figure 2 shows the electrophoretic pattern obtained from preparations before and after adsorption on PAM-2. Lane 3 contains the results obtained when serum-free HUE cell CM is applied directly to the gel. It can be seen that there is a wide variety of proteins present in the preparation and that there is a particularly intense band of about 67 kDA. This protein does not bind to PAM-2–Sepharose (lanes 4 and 5) as would be expected if the protein were some form of tPA. It can be seen from lane 5 that the material which bound to the PAM-2 column shows an enrichment in bands with molecular weights of 105 000, 50 000, and 39 000. Fractions showing activity in the ELISA assay were pooled, concentrated on Amicon Centricon-30 filtration units, and then applied to two linked Du Pont GF-250 columns. Approximately 20 μg of tPA antigenic activity could be recovered from the PAM-2 column for every liter of CM adsorbed. Typically, this would be concentrated to less than 100 μL and be applied to the HPLC column system at a

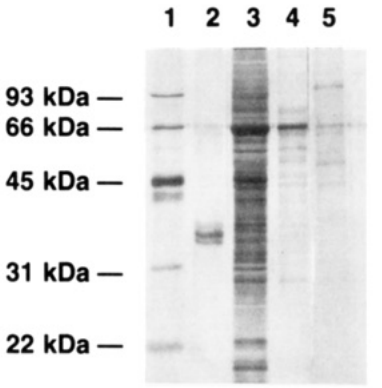

FIGURE 2: Reduced Laemmli gel of PAM-2 fractions. HUE-conditioned medium was examined by electrophoresis both before and after application to a column of PAM-2–Sepharose. Lane 1 contains molecular weight markers; lane 2, two-chain tPA; lane 3, conditioned medium prior to its application to the antibody column; lane 4, material that passed through the PAM-2 column unadsorbed; lane 5, material which bound to the column and which was eluted in buffer containing 2.0 M KSCN. Details on the conditions used for the adsorption of proteins to PAM-2 may be found in the text.

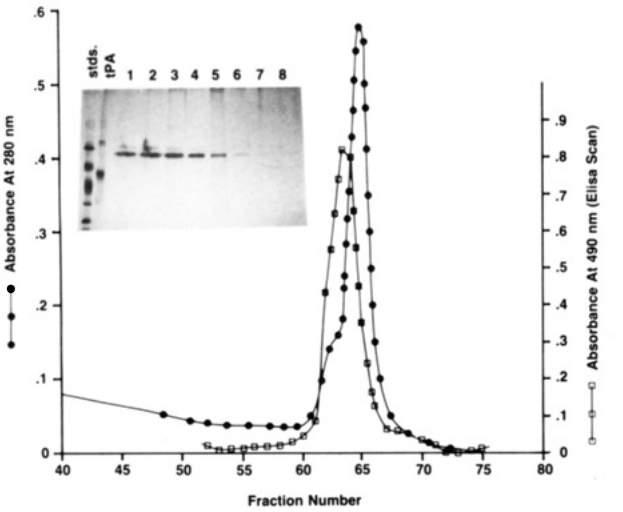

FIGURE 3: HPLC elution profile of PAM-2-purified tPA–inhibitor complexes. tPA–inhibitor complexes were adsorbed on PAM-2–Sepharose and eluted in buffer containing 2.0 M KSCN as described in the text. Fractions showing activity in the tPA ELISA assay were pooled, concentrated on Centricon 30 units, and the applied to two linked GF-250 columns. Columns were run in buffer (0.02 M sodium phosphate, pH 6.8, 1.5 M KSCN, and 0.05% Tween-20) at a flow rate of 1.0 mL/min, and fractions were collected. Aliquots from the fractions were diluted 1:1000 and were scanned in the tPA ELISA assay. Results from the ELISA scan as well as the absorbance profile of the column effluent at 280 nm are shown above. The inset shows the results obtained when aliquots from each fraction were examined on a 5–15% Laemmli nonreduced gel. Lanes 1–8 contain material from fractions 61–68, respectively.

concentration of about 1 μg of tPA μL. The absorbance and ELISA profiles obtained from the columns are shown in Figure 3. The absorbance profile shows a rather broad peak with a shoulder on its leading side. The single peak of tPA ELISA antigenic activity appears to correspond more closely with the shoulder than with the main peak of 280-nm absorbance. Fractions collected at various points along the peak were examined by electrophoresis, and results are shown in the inset of the figure. Lanes 1–8 contain material from fractions 61–68, respectively. It is apparent that each fraction gives an identical pattern, having just one major band with a molecular weight of about 88 000. Also, there appears to be little protein indicated on the right side of the 280-nm peak; instead, the protein detected by silver staining corresponds well with

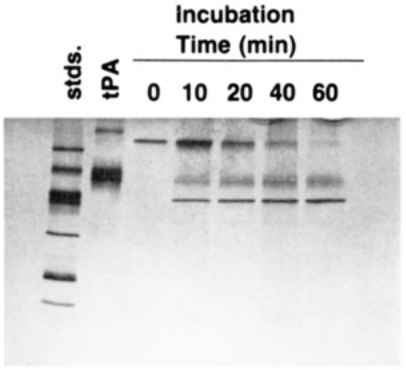

FIGURE 4: Dissociation of tPA–inhibitor complexes by incubation in NH_4OH. Five 100-μL aliquots from fraction 65 (Figure 3) were placed in 1.5-mL Eppendorf tubes. Four of the samples received 100 μL of 3.0 M NH_4OH and were placed in a 37 °C waterbath. The fifth sample received 100 μL of PBS and was left at room temperature. Eppendorf tubes containing NH_4OH were incubated for 10, 20, 40, and 60 min. At the end of these intervals, samples were removed from the incubator and neutralized with HCl. They were examined on a 10–20% Laemmli nonreduced gel, and results are shown above.

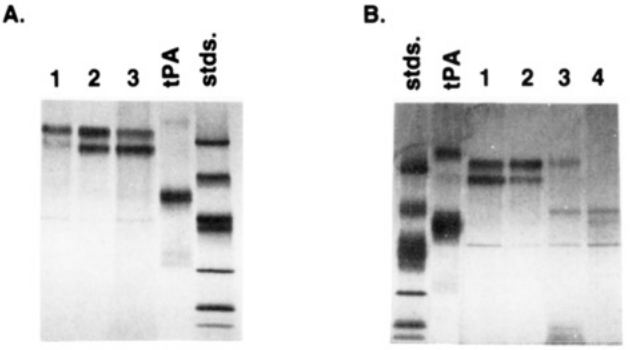

FIGURE 5: Effect of pH and dithiothreitol on the electrophoretic mobility of the tPA–inhibitor complex. 100-μL aliquots from fraction 63 (Figure 3) were incubated under various conditions and then examined on a 10–20% Laemmli gel under nonreduced conditions. Panel A contains results obtained from samples that were incubated at 37 °C for 24 h, and panel B contains results from samples incubated for 48 h. Incubation conditions for the samples in (A) were as follows: lane 1, pH 6.8 + 5 mM dithiothreitol; lane 2, pH 6.8; lane 3, pH 8.0. Incubation conditions for (B) were the following: lane 1, pH 8.0; lane 2, pH 6.8; lane 3, pH 6.8 + 5 mM dithiothreitol; lane 4, pH 6.8 + 5 mM dithiothreitol for 24 h—the pH was then raised to 8.0 and incubation continued for another 24 h.

the tPA antigenic activity determined by the ELISA assay. These results suggest the presence of a nonprotein light-absorbing substance eluting in close proximity to the tPA–inhibitor complex. We have found that when we concentrate buffers which are devoid of protein but which contain either 0.05% Tween-20 or 0.01% Tween-80 and then apply the concentrate to GF-250 columns, we consistently obtain an HPLC absorbance peak that elutes in a position similar to the main peak of absorbance in Figure 3. The displacement in the peak of the ELISA profile from the absorbance peak probably reflects the presence of a similar artifactual light-absorbing substance.

Dissociation and Conformation Studies on Purified tPA–Inhibitor Complexes. Aliquots from fractions 63 and 65 (Figure 3) were used in experiments designed to examine means of dissociating the tPA–inhibitor complex, and it was found that best results could be obtained by incubation in NH_4OH. The results obtained when complex is incubated in 1.5 M NH_4OH at 37 °C for various time intervals are shown in Figure 4. With time, there is a progressive elimination of the high molecular weight tPA–inhibitor complex and the appearance of two major bands: one with a molecular weight indicative of free tPA and the other with a molecular weight of approximately 40 000 (the presumptive inhibitor). It is worth noting that the third lane from the left contains fraction 65 prior to exposure to NH_4OH and that the band in this lane, which previously appeared with a molecular weight of 88 000 (Figure 3, inset, lane 5), has moved to a position indicating a 105 000 molecular weight. This shift in apparent molecular weight occurred between the time the gel filtration columns were run and the subsequent dissociation experiment, a period of several days storage in elution buffer (0.02 M sodium phosphate, pH 6.8, 1.5 M KSCN, and 0.05% Tween-20).

The effect of environmental factors on band position was examined in the experiment whose results are shown in Figure 5A. In this experiment, purified tPA–inhibitor complex was incubated for 24 h at 37 °C at pH 8.0, 6.8, and 6.8 in the presence of 5 mM dithiothreitol (DTT). The pH 8.0 incubation results in a prevalence of the 88-kDa form of the complex (lane 3), whereas incubation at pH 6.8 results in an intensification of the band corresponding to the 105-kDa species (lane 2). In the presence of DTT, the 88-kDa species is almost completely eliminated. The experiment can be extended further with the results shown in Figure 5B. In this experiment, both the pH 8.0 and pH 6.8 incubations have been carried out for 48 h. Results for the pH 8.0 incubation are shown in lane 1, and it can be seen that the two bands are fairly even in intesity, with the 88-kDa species predominating slightly. The pH 6.8 incubation produces a clear predominance of the 105-kDa band (lane 2). After 48 h in the presence of 5 mM DTT, most of the complex has dissociated into tPA and free inhibitor (lane 3). The last lane shows the results which occur when the complex is incubated at pH 6.8 with 5 mM DTT for 24 h and then transferred to buffer at pH 8.0 for 24 h. The raised pH increases the reducing potential of DTT, and in response to this treatment, the dissociation of complexes is nearly complete. Therefore, in addition to NH_4OH, it appears that DTT is capable of separating the complex into its component proteins. It is also important to note that the shift between the 88- and 105-kDa forms of the complex can take place in either direction. For example, in Figure 3 (lane 5), the complex appears as a band with an apparent molecular weight of 88 000. This same material appears as a band with a 105 000 apparent molecular weight in Figure 4 (lane 1) and Figure 5 (lane 1) and after HPLC (Figure 6, inset). The 105-kDa form of the complex was shifted to the 88-kDa form in the experiments described above. The interconversion between these two distinct forms under a variety of conditions and the absence of other forms of the complex suggest that the shifts represent real conformational changes in the proteins involved and are not simply an artifact of the system. There may be a number of other factors which determine whether the 88- or 105-kDa form of the complex predominates in solution; thus far, sample storage conditions, pH, and the reducing potential of the buffer all appear to play a role.

Effect of Fibrin on tPA Enzymatic Activity in Purified tPA–Inhibitor Complexes. tPA enzyme assays (Materials and Methods) were used to determine if fibrin could promote a recovery of plasminogen activator activity from tPA–inhibitor complexes. No proteolytic activity was observed in preparations of complex assayed in the absence of fibrin, and no activity was observed when fibrin alone was assayed. In the presence of fibrin, preparations exhibited an amount of activity equivalent to about 1% of the total activity that would be expected if all the tPA (based on ELISA determinations) were fully active. Therefore, either there is a small portion of the complexes which can be activated under the conditions which were used or the preparations contain a small amount of un-

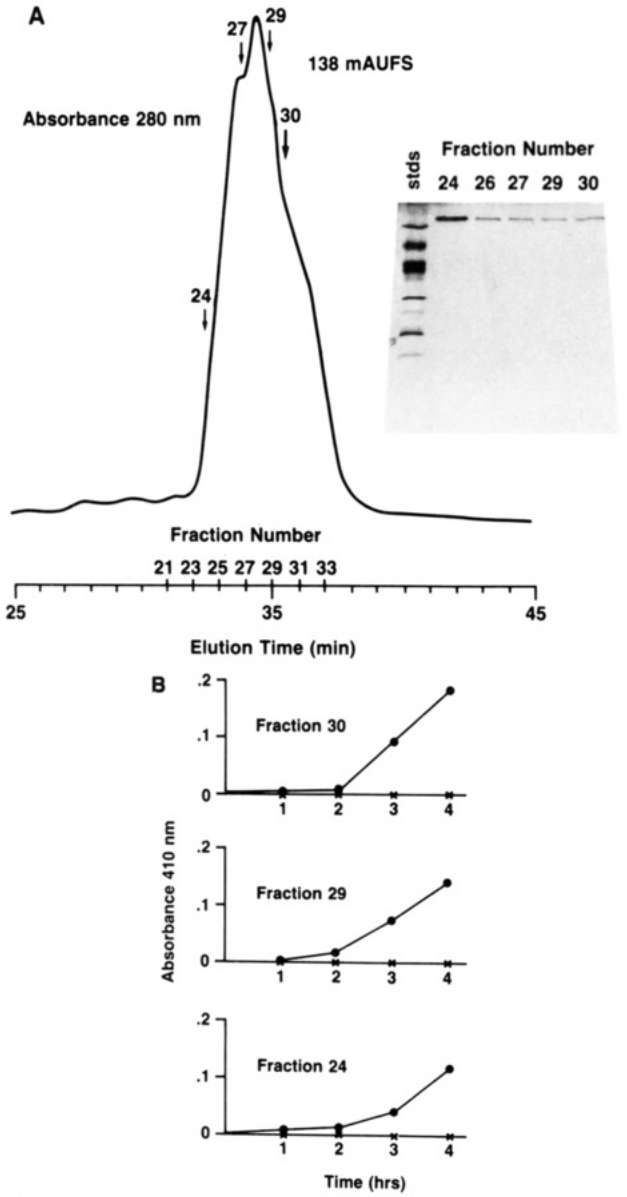

FIGURE 6: Recovery of tPA enzymatic activity from purified preparations of tPA–inhibitor complex. tPA–inhibitor complexes were purified by affinity chromatography and gel filtration as described in the text. The absorbance profile at 280 nm obtained from the HPLC columns is shown in (A). Several fractions (denoted by arrows in the elution profile) were examined by electrophoresis on a 5–15% nonreduced Laemmli gel, and results are shown in the inset of (A). Fraction 24, from the early part of the absorbance peak, and fractions 29 and 30, from a later portion of the peak, were examined in tPA enzyme assays. The assay utilizes a chromogenic substrate which is cleaved at a rate proportional to the amount of tPA present in samples and which produces an increase in the absorbance of solutions at 410 nm (Materials and Methods). Samples were examined both in the absence (×) and in the presence (●) of fibrin, and results are shown in (B).

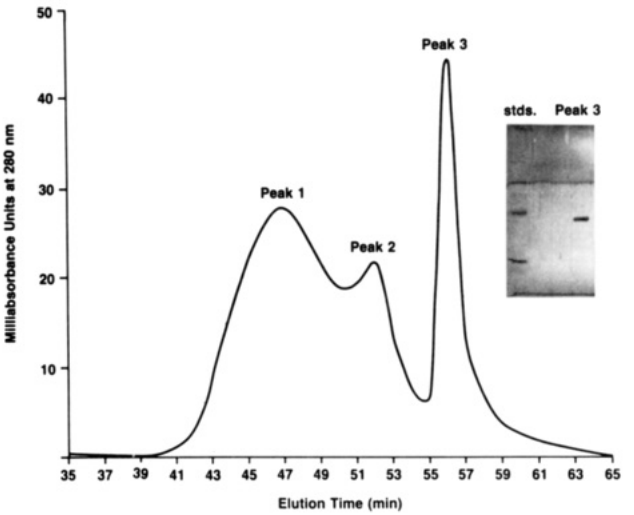

FIGURE 7: Gel filtration of dissociated tPA–inhibitor complexes. Purified tPA–inhibitor complexes were dissociated into their component proteins by incubation in 1.5 M NH_4OH at 37 °C for 1 h. After the preparation was neutralizing with HCl, it was concentrated by using Centricon 10 units and the concentrate applied to three linked GF-250 columns. Columns were run in buffer (0.02 M sodium phosphate, pH 6.8, 1.5 M KSCN, and 0.05% Tween-20), at a flow rate of 0.5 mL/min, and fractions were collected every 0.5 min. The absorbance of the column effluent was monitored at 280 nm and is shown above. The third peak in the profile was well resolved and eluted at a time expected for a protein with a molecular weight of 40 000. Fractions comprising this peak were pooled and examined by electrophoresis under reducing conditions (inset). A single band appears which migrates in a position similar to that observed for the 40-kDa protein in Figure 4.

bound tPA which copurifies with complex. The latter possibility appears unlikely for two reasons: First, the tPA–inhibitor complex in HUE cell CM has been reported to have a dissociation constant of 3.0 pmol/L (Sprengers et al., 1985), and on the basis of experiments in which exogenous tPA was titrated into HUE cell CM and residual activity determined, we estimate that there is about a 5-fold molar excess of inhibitor to tPA antigen in the CM. Even if there had been a 100% recovery of free tPA, the amount present in the samples of purified complex which were assayed should have been considerably lower than the detection limit of our assay. Second, we have obtained HPLC fractions which elute well before the expected elution time for unbound tPA, which appear pure when examined by electrophoresis, and which show tPA activity in the presence of fibrin. An example is shown in Figure 6A,B. It can be seen that fibrin-stimulated proteolytic activity is clearly present in fractions 24 and 29. Both of these fractions elute substantially earlier than the time at which melanoma tPA elutes when chromatographed under identical conditions (36 min).

Isolation of the Dissociated Inhibitor Polypeptide and Partial Sequencing. Preparations of purified tPA–inhibitor complex were dissociated by incubation in NH_4OH, and the mixture of polypeptides was then applied to three linked GF-250 HPLC columns. A typical elution profile is shown in Figure 7. A total of three peaks elute: two overlapping, relatively high molecular weight peaks and a third, well-resolved peak eluting with a retention time similar to that of ovalbumin (57 min). The elution position of the third peak suggests a molecular weight similar to that reported for the complex HUE tPA inhibitor, i.e., about 40 000 (Kruithof et al., 1984; Levin, 1983). SDS electrophoresis confirmed the molecular weight estimate and showed that the polypeptide was in a highly purified state (Figure 7, inset). N-Terminal sequence analysis was performed, and the yield of each amino acid was determined by comparing sample peak heights with the heights of standard PTH-amino acids. At each cycle of Edman degradation, there were two amino acids present in nearly equal molar amounts and a third amino acid present at a much higher concentration. The two equimolar signals appeared to be generated by peptides with identical sequences which differed only in that one contained two additional amino acids at its N-terminus. The amino acids obtained for the first 15 cycles of degradation were grouped into the three sequences shown in Table I. The relative level of each sequence was estimated by determining the yields for the amino acids at each position and then performing linear regression to determine

Table I: Results of N-Terminal Sequence Analysis

amino acid[a] position	sequence 1 (intact N-terminal)	sequence 2 (intact N-terminal extended)	sequence 3 (internal)
1	Val	Ser	Met
2	His	Ala	Ala
3	His	Val	Pro
4	Pro	His	Glu
5	Pro	His	Glu
6	Ser	Pro	Ile
7	Tyr	Pro	Ile
8	Val	Ser	Met
9	Ala	Tyr	Asp
10	His	Val	Arg
11	Leu	Ala	Pro
12	Ala	His	Phe
13	Ser	Leu	Leu
14	Asp	Alu	Phe
15	Phe	Ser	Val

[a] All sequences proceed from the N- to the C-terminus.

initial yields. Using this procedure, the sequences were found to occur at relative molar amounts of 1 to 1 to 5 (for example, 9.25 pmol for sequence 1, 9.29 pmol for sequence 2, and 47.6 pmol for sequence 3). The PAI-1 cDNA from a variety of sources has been isolated, and nucleotide sequences have been reported (Andreasen et al., 1986; Ny et al., 1986; Pannekoek et al., 1986). Comparing the data shown in Table I with these published reports, it can be concluded that sequences 1 and 2 are two different N-terminal arrangements for the intact protein. The deletion of two amino acids from the N-terminus of a portion of PAI-1 sequences has been reported by Andreasen for the inhibitor from fibrosarcoma cells (Andreasen et al., 1986). Since both his source and methods differ substantially from ours, it is unlikely that the heterogeneity at the N-terminus is an artifact generated during our purification procedure. Sequence 3 in Table I is an internal sequence located about 33 amino acids from the C-terminus of the inhibitor protein. Its appearance in our preparations as a free peptide supports the proposal that the inhibitor is cleaved between the Met which begins sequence 3 and the Arg residue which immediately precedes it in the intact protein. The greater molar amount of sequence 3 present in our preparations compared to sequences 1 and 2 suggests that in addition to exhibiting heterogeneity, a large portion of the N-termini of the intact inhibitor may be blocked.

Discussion

The purification procedure presented in this paper was designed to isolate any form of tPA or tPA–inhibitor complex present in HUE cell CM and capable of binding to PAM-2 antibody. Uncomplexed forms of the tPA inhibitor would not be purified. The results that were obtained suggest that virtually all of the tPA in the conditioned medium is bound to a single type of inhibitor protein. It has been previously reported that tPA activity may be partially recovered from tPA–inhibitor complexes after exposure to SDS (Levin, 1983; Stalder et al., 1985; Booth et al., 1985). In these reports, the recovery of activity did not appear to be associated with dissociation of the complex, and it was suggested that the appearance of tPA activity may have been due to a conformational change induced by the detergent. Our results indicate that the complex from endothelial cells does form at least two distinct, interconvertible conformations which may be separated by electrophoresis into forms with apparent molecular weights of 88 000 and 105 000. The fact that these forms remain as distinct entities during the electrophoretic procedure indicates that the conformations must be fairly stable and interconvert only very slowly. Although we have found that there is a small percentage of the total tPA–inhibitor complexes which reexpress proteolytic activity in response to fibrin, we have not yet been able to associate this activity with a particular conformation of the complex. Part of the difficulty in making such an association is that, since a similarly small percentage of complexes are activated by SDS, fibrin overlays cannot be readily used. It will probably be necessary to establish conditions under which a greater portion of the complexes become active before definitive results can be obtained.

It was found that, as might be expected, the tPA–inhibitor complex dissociated in response to incubation in 1.5 M NH_4OH. The dissociation in response to dithiothreitol, however, was quite surprising in that serpins are not generally separated from their target protease by reducing agents. We have found that the effect of dithiothreitol varies considerably from one preparation to the next and seems to be more pronounced in complexes from serum-free CM than in those from serum-containing CM. It may be that dithiothreitol only acts on complexes which have not yet formed a covalent bond or the DTT may cause a conformational change which leads to dissociation. In this regard, it is worth noting that dithiothreitol first promotes the conversion of the 88-kDa form of the complex to the 105-kDa form and then, after prolonged incubation, it promotes dissociation. We have also found that there is a large shift in the HPLC elution position of the complex after brief exposure to DTT (data not shown).

N-Terminal sequencing of the purified 40-kDa inhibitor protein revealed that there were three distinct sequences present. When compared with published reports on the cloned PAI-1 cDNA, it was found that two of the amino acid sequences were located at the N-terminus of the intact protein and the third sequence was found internally. Therefore, it appears that the intact protein has been cleaved and that the cleavage peptide is carried along with the larger fragment during subsequent purification steps. These results are analogous to those that were obtained during the time that the structure of α_1-antitrypsin was being determined (Travis & Salvesen, 1983). α_1-Antitrypsin has a molecular weight of about 53 000 before binding to protease and a weight of less than 50 000 when isolated from protease–inhibitor complexes. As with all the other serpins, the reduction in molecular weight is due to the cleavage of the inhibitor near its C-terminus (Travis & Salvesen, 1983; Carrell & Travis, 1985). This results in the formation of a large inhibitor fragment covalently bound to the protease and a small cleavage peptide. In 1976, Johnson and Travis used protease–inhibitor complexes to isolate the large α_1-antitrypsin fragment and obtained a partial N-terminal sequence (Johnson & Travis, 1976). Other investigators reported that this exact same sequence occurred near the C-terminus of the protein (Carrell et al., 1979; Martodam & Liener, 1981). Subsequent work indicated that the large fragment isolated by Travis had a blocked N-terminal amino acid and that the cleavage peptide had remained attached to the large fragment through strong hydrophobic interactions (Travis & Salvesen, 1983). Therefore, the sequence reported by Travis was not at the N-terminus of the large inhibitor fragment but rather at the protease cleavage site which formed the N-terminus of the cleavage peptide. Since the members of the serpin family which have been characterized thus far are all functionally and structurally very similar, it seems reasonable to use the results obtained for α_1-antitrypsin as a guide in explaining the observations made on the tPA inhibitor. Therefore, it appears that sequence 3

in Table I begins at the N-terminus of the cleavage peptide. The inhibitor is cleaved between Met (the first amino acid in sequence 3) and Arg, the amino acid immediately preceding it in the native inhibitor sequence.

REFERENCES

Andreasen, P. A., Riccio, A., Welinder, K. G., Douglas, R., Sartorio, R., Nielsen, L. S., Oppenheimer, C., Blasi, F., & Dano, K. (1986) *FEBS Lett. 209*, 213.

Bergsdorf, N., Nilsson, T., & Wallen, P. (1983) *Thromb. Haemostasis 50*, 740.

Binder, B. R., Spragg, J., & Austen, K. F. (1979) *J. Biol. Chem. 354*, 1978.

Booth, N. A., Anderson, J. A., & Bennett, B. (1985) *Thromb. Res. 38*, 261.

Brommer, E. J. P., Verheijen, J. H., Chang, G. T. G., & Rijken, D. C. (1984) *Thromb. Haemostasis 52*, 154.

Carrell, R., & Travis, J. (1985) *Trends Biochem. Sci. (Pers. Ed.) 10*, 20.

Carrell, R., Owen, M., Brennan, S., & Vaughan, L. (1979) *Biochem. Biophys. Res. Commun. 91*, 1032.

Chmielewska, J., Ranby, M., & Wiman, B. (1983) *Thromb. Res. 31*, 427.

Collen, D. (1980) *Thromb. Haemostasis 43*, 77.

Colucci, M., Paramo, J. A., & Collen, D. (1985) *J. Clin. Invest. 75*, 819.

Emeis, J. J. (1985) *Thromb. Haemostasis 54*, 230.

Erickson, L. A., Hekman, C. M., & Loskutoff, D. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8710.

Gimbrone, M. A., Jr. (1976) *Prog. Hemostasis Thromb. 3*, 1.

Groves, W. E., Davis, F. C., Jr., & Sells, B. H. (1968) *Anal. Biochem. 22*, 195.

Hamsten, A., Wiman, B., DeFaire, U., & Blomback, M. (1985) *N. Engl. J. Med. 313*, 1557.

Hunkapiller, M. W., Hewick, R. M., Dreyer, R. J., & Hood, L. E. (1983) *Methods Enzymol. 91*, 339.

Jaffe, E. A. (1980) *Transplant. Proc. 12 (Suppl. 1)*, 49.

Johnson, D., & Travis, J. (1976) *Biochem. Biophys. Res Commun. 72*, 33.

Juhan-Vague, I., Moerman, B., DeCock, F., Aillaud, M. F., & Collen, D. (1984) *Thromb. Res. 33*, 523.

Knedler, A., & Ham, R. G. (1983) *In Vitro 19*, 254.

Korninger, C., Wagner, O., & Binder, B. R. (1985) *J. Lab. Clin. Med. 105*, 718.

Kruithof, E. K. O., Tran-Thang, C., Ransijn, A., & Buchmann, F. (1984) *Blood 64(4)*, 907.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Levin, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6804.

Martodam, R. R., & Liener, I. (1981) *Biochim. Biophys. Acta 667*, 328.

Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307.

Ny, T., Sawdey, M., Lawrence, D., Millan, J. L., & Loskutoff, D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6776.

Ogston, D., & Walker, J. E. (1980) *J. Physiol. (London) 303*, 45.

Okamura, T., Nanno, S., Sueishi, K., & Tanaka, K. (1984) *Acta Pathol. Jpn. 34(4)*, 743.

Pannekoek, H., Veerman, H., Lambers, H., Diergaarde, P., Verweij, C. L., van Zonneveld, A.-J., & van Mourik, J. A. (1986) *EMBO J. 58*, 2539.

Ranby, M., Norrman, B., & Wallen, P. (1982) *Thromb. Res. 27*, 743.

Sprengers, E. D., Prinsen, H. M. G., Kooistra, T., & van Hinsbergh, V. W. M. (1985) *J. Lab. Clin. Med. 105*, 751.

Stalder, M., Hauert, J., Kruithof, E. K. O., & Bachman, F. (1985) *Br. J. Haematol. 61*, 169.

Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655.

Verheijen, J. H., Rijken, D. C., Chang, G. T. G., Preston, F. E., & Kluft, C. (1984) *Thromb. Haemostasis 52(3)*, 396.

Wiman, B. (1983) *Clin. Chim. Acta 127*, 279.

Wiman, B., Chmielewska, J., & Ranby, M. (1984a) *J. Biol. Chem. 259(6)*, 3664.

Wiman, B., Csemiczky, G., Marsk, L., & Robbe, H. (1984b) *Thromb. Haemostasis 52*, 124.