Characterization of Recombinant Human α_2-Antiplasmin and of Mutants Obtained by Site-Directed Mutagenesis of the Reactive Site†

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ABSTRACT: Human α_2-antiplasmin (α_2AP) has been expressed in Chinese hamster ovary cells and purified from conditioned media. The recombinant protein (rα_2AP) is immunologically identical with natural α_2AP and indistinguishable with respect to plasmin(ogen) binding properties. Second-order rate constants (k_1) for the interaction of α_2AP and rα_2AP with plasmin are both (1–2) × 10^7 M^{-1} s^{-1}. In order to examine the effects of alterations within the reactive site of α_2AP, deletions of the P_1 residue Arg-364 (rα_2AP-ΔArg364) or the P'_1 residue Met-365 (rα_2AP-ΔMet365) were introduced by in vitro site-directed mutagenesis. rα_2AP-ΔMet365 completely retains its ability to inhibit both plasmin and trypsin, indicating that α_2AP has no absolute requirement for Met in the P'_1 position. Unexpectedly, no increase in antithrombin activity was observed. rα_2AP-ΔArg364 has lost the ability to inhibit plasmin, trypsin, and thrombin, but unlike the wild-type protein, this variant is an effective elastase inhibitor (k_1 = 1.5 × 10^5 M^{-1} s^{-1}).

In plasma, fibrinolysis is regulated by inhibition of both plasminogen activation and plasmin activity. Control is essential for the maintenance of hemostasis.

α_2-Antiplasmin (α_2AP)[1] is the primary physiological plasmin inhibitor (Collen, 1976; Moroi & Aoki, 1976; Müllertz & Clemmensen, 1976). The inhibition is rapid, occurring with a second-order rate constant of (2–4) × 10^7 M^{-1} s^{-1} (Wiman & Collen, 1978). Recently, the complete primary structure of human α_2AP has been deduced from the sequence of its cDNA (Holmes et al., 1987). Alignment of amino acid sequences confirms α_2AP's membership in the serpin (*ser*ine *p*rotease *in*hibitor) superfamily (Lijnen et al., 1982; Carrell & Travis, 1985). The reactive site $P_1P'_1$ sequence of human α_2AP is Arg-364-Met-365 (Holmes et al., 1987). This was deduced from the following observations. The NH_2-terminal amino acid sequence of the M_r 8000 peptide released from the COOH-terminal region of α_2-antiplasmin during its reaction with plasmin is Met-Ser-Leu-Ser (Wiman & Collen, 1979). The COOH-terminal amino acid sequence of the modified inhibitor lacking this M_r 8000 peptide is -Ser-Arg, suggesting that the reactive site $P_1P'_1$ sequence is Arg-Met in the sequence Ser-Arg-Met-Ser-Leu-Ser. Confidence that this amino acid sequence is correct comes from the amino acid sequence predicted by the cloned cDNA (Holmes et al., 1987). Furthermore, there is only one Met-Ser-Leu-Ser sequence in α_2-antiplasmin, and this sequence is preceded by Ser-Arg (Holmes et al., 1987).

The target specificities of the serpins are primarily defined and critically dependent on the P_1 residue, which is located on the carbonyl side of the reactive site peptide bond (Schechter & Berger, 1967). Implication of the P_1 residue as the main determinant of specificity initially came from a comparison of the reactive site sequence of α_1-antitrypsin (α_1AT), which has Met in the P_1 position, with that of antithrombin III, which has Arg as the P_1 residue (Johnson & Travis, 1978; Bjork et al., 1982). α_1AT is primarily an inhibitor of elastase and a poor inhibitor of thrombin (Beatty et al., 1980), whereas antithrombin III (ATIII), the primary thrombin inhibitor, does not inhibit elastase. Further evidence is provided by the Pittsburgh mutant of α_1AT in which the P_1 Met residue has been replaced by natural mutation with Arg, whereby it has lost the ability to inhibit elastase and has become an effective inhibitor of thrombin (Owen et al., 1983; Scott et al., 1986; Travis et al., 1986).

As the reactive sites of other serpins were elucidated, the P_1 residue was observed to generally correspond to the substrate specificity of the cognate serine protease. By extrapolation and with knowledge of the known reactivity of elastases toward various substrates (McRae et al., 1980; Nakajima et al., 1979; Zimmerman & Ashe, 1977), several investigators have exploited the role of the P_1 residue in dictating specificity. Site-directed in vitro mutagenesis of recombinant α_1AT (rα_1AT) was used to construct an oxidation-resistant elastase inhibitor (Courtney et al., 1985; Travis et al., 1985) and several variants differing in specificity and in activity (Jallat et al., 1986).

That amino acids in P subsites other than the P_1 are important in determining inhibitor specificity or in maintaining

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[1] Abbreviations: α_2AP, α_2-antiplasmin; nα_2AP, natural α_2AP, purified from human plasma; rα_2AP, recombinant α_2AP; rα_2AP-ΔArg364, rα_2AP with Arg-364 deletion; rα_2AP-ΔMet365, rα_2AP with Met-365 deletion; α_1AT, α_1-antitrypsin; rα_1AT, recombinant α_1AT; rα_1AT-Ile356-Met358, rα_1AT with the wild-type residues Ile-356 and Met-358; rα_1AT-Ile356-Val358, rα_1AT with Met-358 replaced by Val; rα_1AT-Ala356-Val358, rα_1AT with Ile-356 replaced by Ala and Met-358 by Val; ATIII, antithrombin III; CHO, Chinese hamster ovary; S-2251, D-valylleucyllysine-*p*-nitroanilide (D-Val-Leu-Lys-pNA); S-2484, pyroglutamylprolylvaline-*p*-nitroanilide (Pyro-Glu-Pro-Val-pNA); S-2238, D-phenylalanylpipecolylarginine-*p*-nitroanilide (D-Phe-Pip-Arg-pNA); S-2222, benzoylisoleucylglutamylglycine-*p*-nitroanilide (Bz-Ile-Glu-Gly-Arg-pNA); IU, international unit(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 6-AHA, 6-aminohexanoic acid; *p*-NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; EDTA, ethylenediaminetetraacetate; LBS, lysine binding site; LBSI, plasminogen fragment consisting of kringle structures 1–3 of the plasmin A chain and containing the high-affinity LBS; NIH, National Institutes of Health; KIU, kallikrein inhibitor unit(s); MCIE, modified crossed immunoelectrophoresis; bp, base pair(s); HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; PTH, phenylthiohydantoin.

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an appropriate reactive site conformation is provided by results obtained with a rα_1AT variant containing an Ile → Ala substitution in the P_3 position and a Met → Val substitution in the P_1 position (Jallat et al., 1986). Despite the fact that peptides containing the sequence -Ala-Pro-Val are readily cleaved by neutrophil elastase (Nakajima et al., 1979), rα_1AT-Ala356-Val358 is not an effective inhibitor of neutrophil elastase, whereas rα_1AT-Ile356-Val358 has inhibitory activity comparable to that of rα_1AT-Ile356-Met358 (Jallat et al., 1986).

A natural variant of ATIII containing a Ser → Leu substitution in the P'_1 position has lost thrombin inhibitory activity (Stephens et al., 1985), indicating that P′ subsites can also be important.

In the present study, recombinant human α_2AP (rα_2AP) has been expressed and purified from the conditioned medium of Chinese hamster ovary (CHO) cells. In order to examine the effect of alterations within the reactive site, we have by in vitro site-directed mutagenesis deleted the Met residue in the P'_1 position, thereby constructing an inhibitor (rα_2AP-ΔMet365) whose putative reactive site mimics that of ATIII and that of the Pittsburgh mutant of α_1AT (Arg-Ser as $P_1P'_1$). A second variant was constructed (rα_2AP-ΔArg364) in which the Arg-364 (P_1 residue) is deleted, producing the new sequence Met-364-Ser-365, a putative reactive site $P_1P'_1$ analogous to that of α_1AT. Both variants as well as rα_2AP and natural α_2AP were analyzed for their ability to inhibit plasmin, thrombin, trypsin, and elastase.

Experimental Procedures

Expression Vector Construction. λtPA8 (Nelles et al., 1987b) was obtained by cloning in λgt11, cDNA prepared by reverse transcription of Bowes melanoma cell (Rijken & Collen, 1981) poly(A+) mRNA. λAP73 is a λgt11 cDNA clone of human α_2AP, obtained as described (Holmes et al., 1987). pSV328A is a eukaryotic expression vector containing the SV40 early promoter followed by an M13mp11 polylinker (Messing, 1983) and rabbit β-globin 3′-untranslated sequence providing a polyadenylation signal (Van Heuvel et al., 1986). pSV328A was a generous gift from Dr. M. Van Heuvel (Erasmus University, Rotterdam, The Netherlands).

Figure 1 presents a diagram of pSV328AP73, two M13mp18 intermediate DNAs (M13mp18PAAPint1 and M13mp18PAAPint2), and the expression vectors pPAAP, pPAAP-ΔArg364, or pPAAP-ΔMet365. pSV328AP73 was constructed by ligating the 1725 bp *Eco*RI partial DNA restriction endonuclease fragment of α_2AP from λAP73 into the unique *Eco*RI site of pSV328A. M13mp18PAAPint1 was constructed as follows. pSV328AP73 was digested with *Nco*I, filled in by using Klenow DNA polymerase I and four dNTP's, *Bgl*II-linkered (5′-CAGATCTG), and digested with *Bgl*II and *Hin*dIII. λt-PA8 was digested with *Eco*RI and *Bgl*II. The 231 bp *Eco*RI–*Bgl*II fragment of λt-PA8 (Figure 2) and the 1328 bp *Bgl*II–*Hin*dIII fragment of pSV328AP73 were then ligated into *Eco*RI–*Hin*dIII-digested M13mp18 (Yanisch-Perron et al., 1985). In vitro site-directed mutagenesis (Adelman et al., 1983) employing the single-stranded template of M13mp18PAAPint1 was performed in order to delete the 41 nucleotides between the arginine codon [AGA (−1)] of the t-PA "prepro" sequence and the asparagine codon [AAC (+1)] of α_2AP. The following 28 deoxyoligonucleotide long synthetic DNA fragment *complementary* to the template (coding strand) was used as primer in the mutagenesis:

$$\overset{+1\ \ -1}{5'\text{-ACCTGCTCCTGGTTTCTGGCTCCTCTTC-3'}}$$

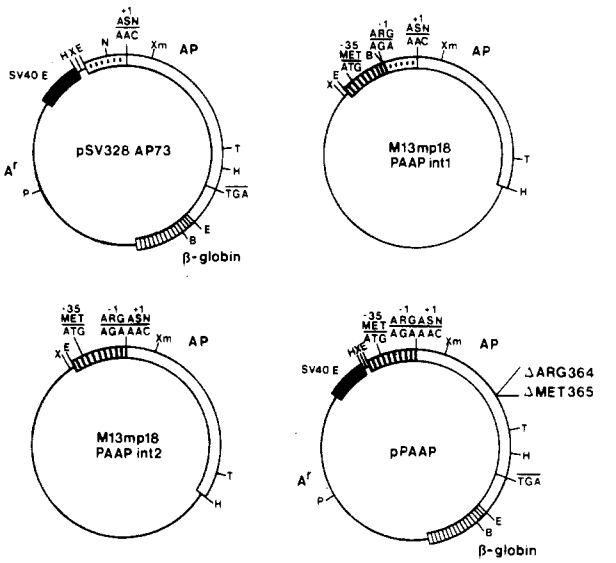

FIGURE 1: Schematic diagram of intermediate plasmids used to construct final expression plasmids pPAAP, pPAAP-ΔArg364, and pPAAP-ΔMet365. Dotted region, sequences 5′ to +1 Asn of α_2AP; thick lines, 5′-untranslated and "prepro" sequence of t-PA; open bars, α_2AP (the +1 Asn residue and the stop codon TGA are indicated); thin lines, rabbit β-globin 3′-untranslated sequence; line, pBR328 sequence. Abbreviations: SV40 E, Simian virus 40 early promoter, H, *Hin*dIII; X, *Xba*I; E, *Eco*RI; N, *Nco*I; T, *Taq*I; B, *Bgl*II; P, *Pst*I; Xm, *Xma*I.

The nucleotide sequence of the spliced DNA in M13mp18PAAPint2 was confirmed by dideoxynucleotide chain-termination sequencing (Sanger et al., 1977).

The final expression vector pPAAP was constructed by four-way ligation using (1) the *Bgl*II–*Eco*RI vector fragment of pSV328AP73, (2) the 327 bp *Eco*RI–*Xma*I fragment of M13mp18PAAPint2, (3) the 1012 bp *Xma*I–*Taq*I fragment of pSV328AP73, and (4) the 749 bp *Taq*I–*Bgl*II fragment of pSV328AP73.

Construction of α_2AP Reactive-Site Variants. In vitro site-directed mutagenesis (Adelman et al., 1983) was performed with the *Xba*I–*Hin*dIII fragment of pPAAP (encoding amino acids Met-35 through Leu-430) cloned in M13mp18 as template. The synthetic complementary deoxyoligonucleotides 5′-GGACAGGGACATGGACATGGCAAT (CGC-Arg-364 deletion) and 5′-GGAGGA-CAGGGAGCGGGACATGGC (ATG-Met-365 deletion) were used as primers. Dideoxynucleotide chain-termination sequencing (Sanger et al., 1977) of the entire *Xba*I–*Hin*dIII fragments verified the desired deletion mutations and confirmed that no other mutations were introduced. These fragments were substituted for the wild-type sequences in pPAAP to yield pPAAP-ΔArg364 or pPAAP-ΔMet365 (Figure 1).

Cell Culture, Transfection, and α_2AP ELISA. CHO dhfr$^-$ cells (Urlaub & Chasin, 1980), a gift from Dr. W. Fiers (University of Gent, Belgium), were maintained in MEMα medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Transfection was performed by using the calcium phosphate coprecipitation method (Graham & van der Eb, 1973) when cells were at about 75% confluency, by contransfection with 0.5 μg of pSVDHFR (Subramani et al., 1981) (American Type Culture Collection 37148) and 5 μg of either pPAAP, pPAAP-ΔArg364, or pPAAP-ΔMet365. DHFR$^+$ cells were selected in HAM's F12 medium without glycine, thymidine, or hypoxanthine and supplemented with 8% dialyzed fetal calf serum. Isolated DHFR$^+$ cells were monitored for secretion of rα_2AP, rα_2AP-ΔArg364, or

rα_2AP-ΔMet365 with an enzyme-linked immunosorbent assay (ELISA) based on three murine monoclonal antibodies to α_2AP according to Holvoet et al. (1986) with the following modifications. Two of the antibodies (MA-34F7 and MA-33B1), directed against different epitopes, were coated on poly(vinyl chloride) microtiter plates (Titertek, Flow, Irvine, Scotland), and after incubation α_2AP. the samples, the third, a horseradish peroxidase (HRP)-conjugated antibody (MA-39A1-HRP), was used to quantitate bound α_2AP. Linear dose–response curves were obtained in the range from 1 to 10 ng/mL. Large-scale production with cell growth on microcarrier beads in roller bottles and serum-free culture conditions were as described (Nelles et al., 1987a). Serum-free conditioned medium from cells previously grown to confluency was harvested after 64 h.

Proteins and Reagents. Native human plasminogen (Glu-plasminogen, M_r 92 000), partially degraded plasminogen (Lys-plasminogen, M_r 85 000), and low molecular weight plasminogen (M_r 39 000) were obtained as previously described (Lijnen et al., 1981). Activation of plasminogen to plasmin was performed by overnight treatment at 4 °C of lysine–Sepharose-bound plasminogen in 0.1 M KH_2PO_4, pH 7.4, with urokinase (1000 IU/mg of plasminogen) followed by elution with 50 mM 6-aminohexanoic acid (6-AHA). Low molecular weight plasminogen was activated to low molecular weight plasmin with streptokinase (1500 IU/mg of protein) in 0.1 M phosphate buffer, pH 7.30, containing 25% glycerol at 0 °C. The concentration of plasmin or low molecular weight plasmin was determined by active-site titration using *p*-nitrophenyl *p*-guanidinobenzoate (*p*-NPGB) (Chase & Shaw, 1970); at least 95% activation was obtained.

Human thrombin (M_r 37 000) was obtained as a gift from Dr. J. W. Fenton (State Department of Health, Albany, NY); it was 92% active as determined by active-site titration using *p*-NPGB. Bovine trypsin (M_r 22 500) was obtained by activation of trypsinogen (Boehringer Mannheim) (50 μM) at pH 8.0 with porcine enterokinase (Sigma, St. Louis, MO) (0.7 nM) at 4 °C for 24 h (Maroux et al., 1971), and its activity was determined by active-site titration using *p*-NPGB. Elastase (M_r 30 000) from human sputum was purchased from Elastin Products Co. (Pacific, MO). The protein concentration was measured spectrophotometrically using $A_{1\,cm}^{1\%} = 9.85$ at 280 nm, and the protein was shown to be fully active by titration with human α_1AT.

Human α_1AT (M_r 54 000), purified from human plasma (Laurell et al., 1975), was titrated against bovine trypsin, and its activity amounted to 75% of the protein.

Human ATIII (M_r 60 000) was purified from plasma by affinity chromatography on heparin–Ultrogel (Miller-Andersson et al., 1974) and titrated against thrombin.

Natural α_2AP (nα_2AP) (M_r 67 000) was purified from human plasma by chromatography on LBSI–Sepharose (Wiman, 1980) and DEAE-Sephadex A-50 (Wiman & Collen, 1977). Its active concentration was determined by titration against plasmin of known concentration (Wiman & Collen, 1978) and amounted to more than 95% of the protein.

All measurements of enzyme and inhibitor activities were performed at 25 °C in 0.05 M Tris·HCl buffer, pH 7.40, containing 0.038 M NaCl and 0.01% Tween 80. The chromogenic substrates S-2251 (D-Val-Leu-Lys-pNA), S-2484 (Pyro-Glu-Pro-Val-pNA), S-2222 (Bz-Ile-Glu-Gly-Arg-pNA), and S-2238 (D-Phe-Pip-Arg-pNA) were purchased from KabiVitrum (Brussels, Belgium). The plasmin inhibitor D-Val-Phe-Lys-CH_2Cl was custom synthesized at Union Chimique Belge (Brussels, Belgium). Rabbit antiserum (polyclonal) against human α_2AP was obtained as previously described (Collen, 1976).

Purification of rα_2AP's. Serum-free conditioned media (750–1500-mL batches) were concentrated 5–10-fold by ultrafiltration on Amicon PM10 membranes and applied at 4 °C to a LBSI–Sepharose column (0.9 × 7 cm for rα_2AP and rα_2AP-ΔArg364 and 2.5 × 9 cm for rα_2AP-ΔMet365) equilibrated with 0.05 M phosphate buffer, pH 7.0, at a flow rate of about 10 mL/h. The columns were subsequently washed with (1) 0.05 M phosphate buffer, pH 7.0, (2) the same buffer containing 0.5 M NaCl, and (3) again with 0.05 M phosphate buffer, pH 7.0. Elution was performed with 10 mM 6-AHA in 0.05 M phosphate buffer, pH 7.0. The protein peak was pooled, concentrated 5–10-fold by ultrafiltration on Amicon PM10, and applied at 4 °C to DEAE-Sephadex A-50 (0.9 × 7 cm for rα_2AP and rα_2AP-ΔArg364 and 2.5 × 6 cm for rα_2AP-ΔMet365) equilibrated in 0.05 M phosphate buffer, pH 7.0, at a flow rate of 5 or 20 mL/h. Elution was performed with a linear gradient from 0 to 0.4 M NaCl (4 column volumes each) in the same buffer. α_2AP–antigen was monitored by electroimmunoassay (Laurell, 1966). The protein was pooled, concentrated, and stored frozen at −20 °C in aliquots. Final protein concentrations were determined by ELISA and by amino acid analysis. Activity was measured by titration against plasmin of known concentration, as described for nα_2AP. This yielded activities of 60% and 100% for two subsequent preparations of rα_2AP and 85% and 86% for rα_2AP-ΔMet365, while no plasmin inhibition was detected with rα_2AP-ΔArg364 under these conditions.

Kinetic Analysis. All kinetic measurements were performed at 25 °C in 0.05 M Tris·HCl buffer, pH 7.40, containing 0.038 M NaCl and 0.01% Tween 80 with at least two independent α_2AP preparations.

Second-order rate constants (k_1) were obtained as follows. (1) Pseudo-first-order conditions: for each inhibitor concentration used, the half-life ($t_{1/2}$) is determined from a linear semilogarithmic plot of residual enzyme activity (measured with a chromogenic substrate) vs. time. k_1 is then obtained by dividing the apparent rate constant ($k_{app} = \ln 2/t_{1/2}$) by the inhibitor concentration. (2) Second-order conditions with equimolar active concentrations of enzyme and inhibitor: k_1 is determined as the slope of a linear plot of $1/[E]$ vs. time, according to the equation (Beatty et al., 1980) $1/[E] = k_1 t + 1/[E]^0$ where $[E]^0$ is the initial enzyme concentration and [E] the concentration of free enzyme measured at different time points using a chromogenic substrate. (3) Second-order conditions with slight excess of inhibitor over enzyme: residual enzyme is measured as a function of time with a chromogenic substrate and k_1 calculated from a standard equation for a second-order reaction:

$$k_1 t = \frac{1}{[I]^0 - [E]^0} \ln \frac{[E]^0([I]^0 - [E]^0 + [E])}{[I]^0[E]}$$

with $[I]^0$ and $[E]^0$ the initial concentrations of inhibitor and enzyme, respectively, and [E] the enzyme concentration at time t.

Inhibition of Plasmin. Inhibition of 8 nM plasmin was measured with 8–11 nM nα_2AP, rα_2AP, or rα_2AP-ΔMet365. The effect of 1 mM 6-AHA on the inhibition rate was also evaluated. Inhibition of 5 nM low molecular weight plasmin was measured with 25–75 nM nα_2AP or rα_2AP. Neutralization of 100 nM plasmin was measured with 0.5–1.5 μM ATIII and with 5–15 μM α_1AT using a plasmin concentration of 200 nM. Residual enzyme activity in all experiments was measured with S-2251 (final concentration 0.3 mM). Inhib-

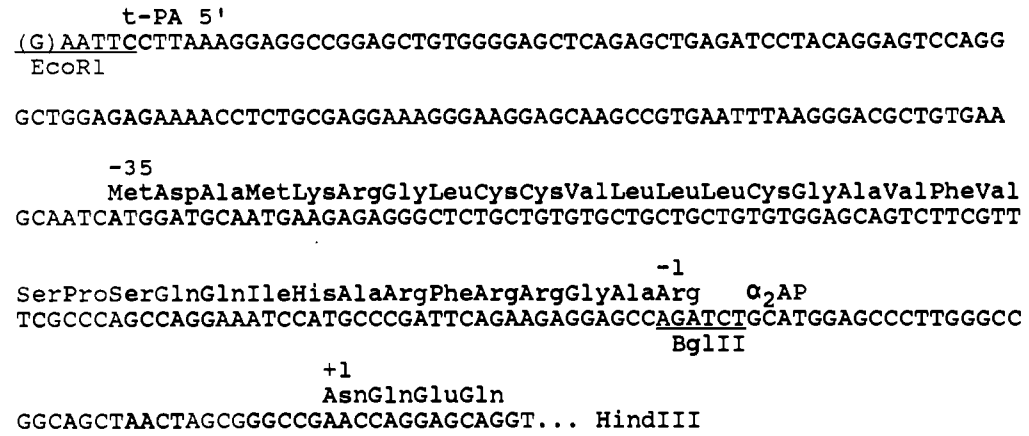

FIGURE 2: Partial nucleotide sequence of the combined coding strands and α_2AP 5′ sequences prior to fusion of the −1 Arg codon of t-PA to the +1 Asn codon of mature α_2AP (details under Experimental Procedures). The 231 bp *Eco*RI–*Bgl*II fragment contains the 5′-untranslated and "prepro" of λt-PA8/cDNA. The *Bgl*II–*Hin*dIII fragment of α_2AP was obtained from pSV328AP73 (Figure 1) by *Nco*I digestion, *Bgl*II-linkering, and *Hin*dIII digestion.

ition of 2 nM plasmin by rα_2AP-ΔArg364 at a 10-fold molar excess was monitored continuously over 30 min in the presence of 0.6 mM S-2251.

Interaction of plasmin with the different α_2AP moieties was also monitored on SDS–PAGE. Therefore, 10 μM plasmin was incubated for 1 min at 37 °C with a slightly lower concentration of α_2AP, followed by addition of 2×10^{-4} M D-Val-Phe-Lys-CH_2Cl and electrophoresis on 12% SDS–PAGE under nonreducing conditions.

Inhibition of Thrombin. Inhibition of 20 nM thrombin was measured under pseudo-first-order conditions, using the following final concentrations of inhibitor: 0.36–1.5 μM for ATIII, 0.2–1 μM for nα_2AP, 0.33–0.84 μM for rα_2AP, 6–10 μM for rα_2AP-ΔMet365, and 9–16 μM for α_1AT. Residual enzyme activity was measured with 0.3 mM S-2238 after 10–20-fold dilution of the samples. Inhibition of 20 nM thrombin preincubated at 37 °C for 5 min with 20 nM high-affinity heparin was measured by using a 50-fold molar excess of rα_2AP-ΔMet365. Inhibition of 2 nM thrombin by a 10-fold molar excess of rα_2AP-ΔArg364 was monitored continuously over 20 min with 0.2 mM S-2238.

Inhibition of Trypsin. Inhibition of 10–40 nM trypsin was measured with equimolar concentrations of α_1AT, nα_2AP, rα_2AP, or rα_2AP-ΔMet365 or with an excess of ATIII (50–150 nM). Residual enzyme activity was measured with 0.2 mM S-2222 after 10–20-fold dilution. Inhibition of 1 nM trypsin by rα_2AP-ΔArg364 at a 10-fold molar excess was monitored continuously over 20 min with 0.4 mM S-2222.

Inhibition of Elastase. Inhibition of 20 nM elastase by 22 nM α_1AT was measured with 0.27 mM S-2484. With nα_2AP, rα_2AP, rα_2AP-ΔMet365, or ATIII, no significant elastase inhibition was obtained within 60 min using a 5–25-fold molar excess of inhibitor. Inhibition of 5 nM elastase by 20–112 nM rα_2AP-ΔArg364 was monitored continuously in the presence of 0.27 mM S-2484. The effect of the presence of substrate on the inhibition rate was corrected for by using the formula (Hoylaerts et al., 1984):

$$k_{app} = \frac{\ln 2}{t_{1/2}}(1 + [S]^0/K_m)$$

with K_m = 0.27 mM as determined by Lineweaver–Burk analysis.

Binding to Fibrin. Normal human plasma or α_2AP-depleted plasma reconstituted with nα_2AP or rα_2AP to normal concentration was clotted for 1 h at 37 °C by addition of a mixture of 25 mM Ca^{2+}, thrombin (final concentration 1.5 NIH units/mL), and 15 mM NaCl. Controls included samples with addition of EDTA (final concentration 10 mM). After centrifugation, residual α_2AP antigen was measured in the supernatant by ELISA.

Other Laboratory Methods. Amino acid analysis was performed on a Beckman 119CL amino acid analyzer after sample hydrolysis in 6 M HCl in vacuo at 110 °C for 20 h. Double immunodiffusion analysis was performed according to Ouchterlony (1958) using rabbit antiserum against human nα_2AP.

α_2AP-depleted human plasma was obtained by immunoadsorption on insolubilized monoclonal antibody MA34F7 (Holvoet et al., 1986). The remaining α_2AP level was about 1% as measured with the ELISA described above.

Crossed immunoelectrophoresis with or without addition of Lys-plasminogen to the agarose gel during electrophoresis in the first dimension was performed as described (Kluft & Los, 1981). α_2AP samples containing ~200 ng (3 μL) were applied.

SDS–PAGE was performed without reduction on 12% gels according to Laemmli (1970). NH_2-terminal amino acid sequence analysis was performed on ~85 pmol of rα_2AP and ~400 pmol of rα_2AP-ΔMet365 by automated Edman degradation (Hunkapiller et al., 1983) (courtesy of Dr. H. Rodriguez, Genentech Inc., South San Francisco) using an Applied Biosystems Inc. (ABI) Vapor Phase Model 470A. Phenylthiohydantoins (PTH) were analyzed on-line by using an ABI 120A PTH–amino acid analyzer.

RESULTS

Production of rα_2AP and rα_2AP Variants. Figure 2 shows the nucleotide and deduced amino acid sequence of the t-PA 5′-untranslated and "prepro" sequence and the α_2AP sequence to which it was ligated in M13mp18PAAPint1. The coding sequence of t-PA is identical with that earlier reported (Pennica et al., 1983), and the 5′-untranslated DNA is extended by 51 nucleotides (Nelles et al., 1987b). Eukaryotic expression vectors were constructed in which the SV40 early promoter directs the transcription of the cDNA of the "prepro" sequence of t-PA, fused to the cDNA encoding human α_2AP or the two variants of α_2AP in which either the codon for the P_1 residue (Arg-364) has been deleted (rα_2AP-ΔArg364) or the codon for the P'_1 residue (Met-365) has been deleted (rα_2AP-ΔMet365) by in vitro site-directed mutagenesis. A polyadenylation signal is provided by rabbit β-globin 3′-untranslated sequence (Figure 1). Secretion of rα_2AP or the variants by CHO cells, cotransfected with pDHFR5 and pPAAP, pPAAP-ΔArg364, or pPAAP-ΔMet365, is thus

Table I: Amino Acid Compositions of α_2-Antiplasmin Moieties[a]

amino acid	sequence	nα_2AP	rα_2AP
Asx	35	36	39
Thr	22	22	23
Ser	35	35	34
Glx	62	66	64
Pro	37	42	38
Gly	26	27	32
Ala	29	28	31
Val	28	20	24
Met	10	10	7
Ile	9	6	6
Leu	67	65	58
Tyr	4	4	6
Phe	28	29	28
His	12	11	10
Lys	19	18	20
Arg	19	17	18

[a] The values represent means of two or three analyses. The number of amino acids is normalized to 442, which is the total number in the sequence (Holmes et al., 1987), excluding Trp and Cys (which were not determined).

mediated by a heterologous signal peptide.

Purification and Physicochemical Characterization. rα_2AP, rα_2AP-ΔMet365, and rα_2AP-ΔArg364 were obtained with average final yields of 35%, 38%, and 20%, respectively.

SDS–PAGE under nonreducing conditions shows homogeneous protein bands (Figure 3). Under reducing conditions (not shown), these proteins migrate with apparent molecular weights of 72 000 for nα_2AP and 81 000 for the three recombinant species. Double immunodiffusion analysis (Figure 4) shows complete immunological identity between the natural and the recombinant proteins. Table I shows that the amino acid compositions of nα_2AP and rα_2AP are indistinguishable. The single amino acid deletions in the variants cannot be detected by amino acid analysis.

NH_2-terminal amino acid sequence analysis of rα_2AP and rα_2AP-ΔMet365 (Table II) shows that the NH_2 terminus of mature nα_2AP (Asn-Gln-Glu-Gln) (Wiman & Collen, 1979) is preceded by three residues, NH_2-Gly-Ala-Arg, corresponding to the COOH-terminal three amino acids of the t-PA sequence employed to secrete α_2AP.

Interactions with Fibrin and Plasminogen. (*A*) *Binding to Fibrin.* Binding of α_2AP to fibrin in reconstituted α_2AP-depleted plasma amounted to 30.3% ± 3% (n = 4) for nα_2AP. No binding was detected with rα_2AP (n = 4). Binding of α_2AP in normal undepleted human plasma equalled 20.3% ± 3.8% (n = 4). In the presence of EDTA, no cross-linking of α_2AP to fibrin was detected.

(*B*) *Interaction with Plasminogen.* Interaction of rα_2AP with plasminogen, suggested by its binding to LBSI–Sepharose, was confirmed by MCIE in the presence of Lys-plasminogen. The electrophoretic mobility (α_2 region) in the absence of plasminogen is identical (Figure 5A) for nα_2AP in plasma or after purification and for rα_2AP in conditioned medium and in purified form. Addition of Lys-plasminogen (Figure 5B) results in a decrease of the mobility of about two-thirds of nα_2AP in plasma and of all of the purified nα_2AP, due to interaction with plasminogen. rα_2AP in the conditioned medium seems to consist mainly of the plasminogen binding form, and purified rα_2AP interacts nearly quantitatively with plasminogen.

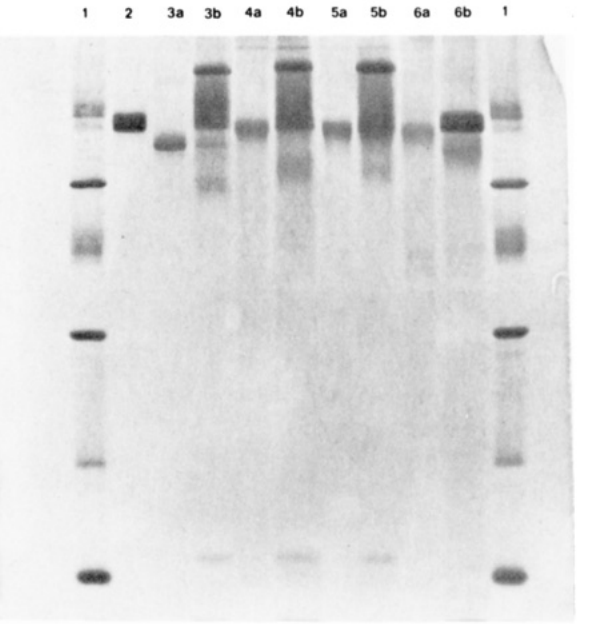

FIGURE 3: SDS–PAGE under nonreducing conditions of α_2AP moieties before (a) and after (b) reaction with a slight molar excess of plasmin for 1 min at 37 °C. (3) nα_2AP; (4) rα_2AP; (5) rα_2AP-ΔMet365; (6) rα_2AP-ΔArg364. Lane 1 shows a protein calibration mixture consisting of phosphorylase *b* (M_r 94 000), albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 000), and α-lactalbumin (M_r 14 400). Lane 2 shows the plasmin preparation used.

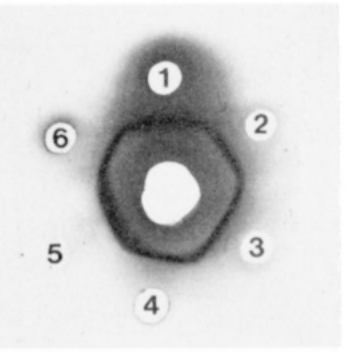

FIGURE 4: Double immunodiffusion. Double immunodiffusion of α_2AP moieties against rabbit anti-human nα_2AP antiserum. (1) Normal plasma; (2) rα_2AP; (3) rα_2AP-ΔMet365; (4) rα_2AP-ΔArg364; (5) nα_2AP; (6) concentrated conditioned cell culture medium of rα_2AP.

Interaction with Enzymes. Table III summarizes the kinetic constants obtained for the inhibition of human plasmin, human thrombin, bovine trypsin, and human sputum elastase by the four α_2AP moieties and by ATIII and α_1AT.

nα_2AP, rα_2AP, and rα_2AP-ΔMet365 appear to be equally potent inhibitors of plasmin (k_1 between 1.7×10^7 and 3.6×10^7 M^{-1} s^{-1}) and of trypsin (k_1 between 2.2×10^6 and 3.5×10^6 M^{-1} s^{-1}). Both nα_2AP and rα_2AP inhibit thrombin with $k_1 \sim 2 \times 10^3$ M^{-1} s^{-1} while rα_2AP-ΔMet365 is much less effective ($k_1 = 0.074 \times 10^3$ M^{-1} s^{-1}). None of the α_2AP

Table II: NH_2-Terminal Amino Acid Sequence of rα_2AP and rα_2AP-ΔMet365

cycle amino acid	1 Gly	2 Ala	3 Arg	4 Asn	5 Gln	6 Glu	7 Gln	8 Val	9 Ser	10 Pro	11 Leu	12 Thr	13 Leu
rα_2AP yield[a]	80	68	44	40	44	32	36	24	24	44	32	24	28
rα_2AP-ΔMet365 yield[a]	400	520	456	336	364	312	312	264	264	260	308	144	300

[a] Yields obtained at each cycle are expressed in picomoles and were obtained from analysis of 85 or 400 pmol of starting material for rα_2AP and rα_2AP-ΔMet365, respectively.

Table III: Enzyme–Inhibitor Reaction Second-Order Rate Constants[a]

inhibitor	$P_1P'_1$ sequence	human plasmin	human thrombin	bovine trypsin	human sputum elastase
α_2AP	Arg-Met	$(1.7 \pm 0.1) \times 10^7$	$(2.5 \pm 0.2) \times 10^3$	$(3.5 \pm 0.2) \times 10^6$	ND
rα_2AP	Arg-Met	$(2.1 \pm 0.1) \times 10^7$	$(1.6 \pm 0.1) \times 10^3$	$(2.5 \pm 0.4) \times 10^6$	ND
rα_2AP-ΔMet365	Arg-Ser	$(3.6 \pm 0.1) \times 10^7$	$(0.07 \pm 0.001) \times 10^3$	$(2.2 \pm 0.2) \times 10^6$	ND
rα_2AP-ΔArg364	Met-Ser	ND[b]	ND	ND	$(3.5 \pm 0.3) \times 10^5$
ATIII	Arg-Ser	$(2.4 \pm 0.3) \times 10^3$	$(3.4 \pm 0.1) \times 10^3$	$(1.4 \pm 0.1) \times 10^5$	ND
α_1AT	Met-Ser	$(1.9 \pm 0.1) \times 10^2$	$(0.03 \pm 0.002) \times 10^3$	$(2.2 \pm 0.3) \times 10^5$[c]	$(1.5 \pm 0.1) \times 10^7$

[a] The data represent k_1 (M^{-1} s^{-1}) and are means ± SD of three or four independent experiments. [b] ND, not detectable inhibition with a 10–25-fold molar excess of inhibitor. [c] $k_1 = 4.2 \times 10^4$ M^{-1} s^{-1} with porcine trypsin (Travis et al., 1985).

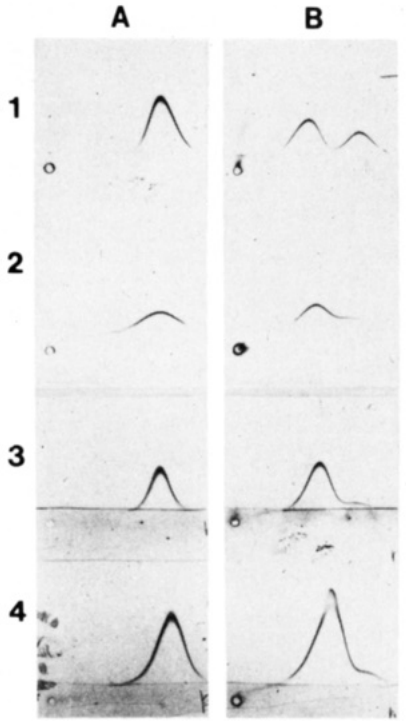

FIGURE 5: Crossed immunoelectrophoresis. (A) Without Lys-plasminogen added to the gel during the first electrophoresis. (B) With Lys-plasminogen present during the first electrophoresis. Crossed immunoelectrophoresis of normal human plasma (1); concentrated conditioned cell culture medium (2); nα_2AP (3); and rα_2AP (4). Electrophoresis in the second dimension was performed after addition of rabbit anti-human nα_2AP antiserum.

moieties nor ATIII inhibits elastase. rα_2AP-ΔArg364 at a 10-fold molar excess does not inhibit any of the enzymes at a significant rate, except elastase which is neutralized with $k_1 = 3.5 \times 10^5$ M^{-1} s^{-1}.

The similarity between nα_2AP and rα_2AP is further confirmed by our findings that addition of 1 mM 6-AHA reduces (20–30-fold) the inhibition rate constant of plasmin by nα_2AP [$k_1 = (6.9 \pm 0.1) \times 10^5$ M^{-1} s^{-1}; $n = 2$] and by rα_2AP [$k_1 = (7 \pm 0.9) \times 10^5$ M^{-1} s^{-1}; $n = 2$] and that the inhibition rates of low molecular weight plasmin by nα_2AP [$k_1 = (3.1 \pm 0.1) \times 10^5$ M^{-1} s^{-1}; $n = 3$] and by rα_2AP [$k_1 = (3.1 \pm 0.1) \times 10^5$ M^{-1} s^{-1}; $n = 2$] are identical.

Inhibition of plasmin by rα_2AP and rα_2AP-ΔMet365 results in the formation of a plasmin–α_2-antiplasmin complex with concomitant release of a peptide from α_2AP with apparent M_r ~14 000. No complex formation is observed with rα_2AP-ΔArg364 (Figure 3).

Discussion

In this study, we have characterized recombinant human α_2-antiplasmin (rα_2AP) purified from the conditioned medium of CHO cells transfected with a plasmid directing rα_2AP synthesis under control of the SV40 early promoter. The effect of deletions of either the P_1 or the P'_1 residues of the reactive site of rα_2AP was also investigated.

Because in the original cloning of the cDNA encoding human α_2AP a complete signal peptide preceded by an initiation codon was not obtained (Holmes et al., 1987), it was necessary to splice DNA encoding a heterologous signal peptide and initiation codon to the NH_2-terminal end of the mature α_2AP cDNA in order to obtain secretion of rα_2AP's. Therefore, in vitro site-directed deletion mutagenesis was used to position the cDNA encoding the 35 amino acid "prepro" sequence of human t-PA. NH_2-terminal amino acid sequence analysis revealed that the secreted rα_2AP has an NH_2-terminal extension of three amino acids when compared to natural α_2AP. Thus, it appears that a processing enzyme has failed to recognize the Arg–Asn peptide bond between the prosequence of t-PA and mature α_2AP and instead has cleaved the Arg–Gly peptide bond three positions NH_2 terminally (Figure 2). Under reducing conditions, rα_2AP migrates with an apparent molecular weight of ~81 000 when visualized after SDS–PAGE. The difference in molecular weight between rα_2AP and nα_2AP (M_r 72 000) is likely due to a difference in glycosylation, because their amino acid compositions are indistinguishable.

The kinetics of the inhibition of plasmin by α_2AP have been well characterized (Christensen & Clemmensen, 1977, 1978; Wiman & Collen, 1978) and are compatible with a kinetic model of two successive reactions. The first is a very fast reversible reaction [$k_1 = (2–4) \times 10^7$ M^{-1} s^{-1}] (Wiman & Collen, 1978) composed of two steps: (1) a reaction between the lysine binding site(s) in the plasmin A chain (Rickly & Otavsky, 1975; Wiman et al., 1979) and corresponding site(s), which are probably located within the COOH-terminal 26 amino acids of α_2AP (Sasaki et al., 1983); (2) a reaction between the active site of plasmin and the reactive site of α_2AP to form a Michaelis-type complex. The second reaction is much slower and involves cleavage of the reactive-site peptide bond in α_2AP. Despite the slight NH_2-terminal difference between nα_2AP and rα_2AP, their k_1 for inhibition of plasmin, their interaction with the lysine binding site(s) of plasmin, and their ability to form tight complexes with plasmin are very similar. nα_2AP and rα_2AP are indeed equally potent inhibitors of plasmin [$k_1 = (1.7–3.5) \times 10^7$ M^{-1} s^{-1}]. Addition of 1 mM 6-AHA (a lysine analogue) reduces their inhibition rates of plasmin about 30-fold. The inhibition rates of low molecular weight plasmin, a form of plasmin lacking the first four kringles containing the lysine binding site(s) (Sottrup-Jensen et al., 1977), are similarly reduced. Further evidence for the interaction with the lysine binding site(s) comes from the affinity for LBSI–Sepharose and from the results of crossed immunoelectrophoresis in the presence of Lys-plasminogen.

Binding of α_2AP to fibrin following clotting of reconstituted α_2AP-depleted plasma in the presence of Ca^{2+} was observed only with nα_2AP. The three additional amino acids on the NH_2 terminus of rα_2AP must have interfered with its cross-linking to fibrin, which is mediated via the Gln residue in position 2 of α_2AP (Ichinose et al., 1983).

α_2AP is a member of the serine protease inhibitor (serpin) superfamily of proteins, most of which are vital regulators of serine proteases. In the majority of instances, a serine is found in the P'_1 position of the reactive sites, and a hydrophobic amino acid is found in the P'_2 position. The reactive-site region is flanked by blocks of nearly invariant residues. The high degree of amino acid homology among members of the superfamily predicts a significant degree of tertiary structure homology. X-ray crystallographic studies of human α_1AT (Loberman et al., 1984) cleaved at its reactive-site peptide bond suggest that the reactive center is contained in what must be a highly strained loop, situated on the surface of the molecule and thus exposed to proteolytic attack.

Although it has been extensively investigated, the exact mechanism of serpin inhibition is not entirely understood. It is known that the inhibitors act by presenting the reactive site (a substratelike region) to their target enzymes, allowing direct interaction. Inhibition occurs as the consequence of a 1:1 stoichiometric interaction involving the active-site serine hydroxyl group of the protease and the carbonyl carbon atom of the P_1 residue. The resulting covalent tetrahedral complex is then hydrolyzed, resulting in cleavage of the $P_1P'_1$ peptide bond and the likely formation of an acyl-enzyme intermediate associated with the release of an amine (Travis & Salvesen, 1983). The exact structure of the linkage between the enzyme and the now modified inhibitor has not been ascertained, however, but it is well-known that the complexes are stable in SDS and unlike the small plant inhibitors (Travis & Salvesen, 1983) the reactive-site cleaved form of the inhibitors is inactive. As a result of this type of complex formation, it has been possible to delineate the reactive-site sequences of a number of serpins by amino acid sequencing of released COOH-terminal peptides, complexes, and overlapping peptides (Johnson & Travis, 1978; Bjork et al., 1982; Moroi & Travis, 1983; Salvesen et al., 1985; Holmes et al., 1987).

Individual serpins interact with several proteases, but often there is a primary inhibitor for each enzyme. Their specificities are generally defined by the P_1 residue (Carrell & Travis, 1985). Neither α_2AP, $P_1P'_1$ Arg-Met, nor ATIII $P_1P'_1$ Arg-Ser inhibits elastase. In the absence of heparin, both inhibit thrombin [$k_1 = (2\text{–}4) \times 10^3$ M^{-1} s^{-1}]. Bovine trypsin is neutralized by α_2AP with $k_1 = \sim 3 \times 10^6$ M^{-1} s^{-1} and by ATIII with $k_1 = \sim 1.4 \times 10^5$ M^{-1} s^{-1}. A significant difference is observed in these two serpins' ability to inhibit plasmin. α_2AP, the primary plasmin inhibitor, inhibits plasmin 10^4-fold faster than does ATIII: $k_1 = 1.6 \times 10^7$ and 2.4×10^3 M^{-1} s^{-1}, respectively. In contrast, α_1AT with $P_1P'_1$ Met-Ser is a highly effective anti-elastase ($k_1 = 1.4 \times 10^7$ M^{-1} s^{-1}), is a very poor inhibitor of plasmin and thrombin, and possesses antitrypsin activity somewhat less than α_2AP and ATIII. An experiment of nature demonstrated by the Pittsburgh mutant of α_1AT in which the P_1 Met residue has been replaced by Arg (Owen et al., 1983) results in a dramatic loss of anti-elastase activity, an equally dramatic gain in antithrombin activity ($k_1 = 3.1 \times 10^5$ M^{-1} s^{-1}) (Travis et al., 1986; Scott et al., 1986), a 1000-fold increase in antiplasmin activity ($k_1 = 2 \times 10^5$ M^{-1} s^{-1}) (Carrell & Boswell, 1986), and a 10–100-fold increase in antitrypsin activity ($k_1 = 3.0 \times 10^6$ M^{-1} s^{-1} for porcine trypsin). Several groups have shown that by substituting other amino acids for the P_1 Met of rα_1AT, a wide range of inhibitory activities differing in specificity may be obtained (see the introduction).

Evidence that P′ subsite amino acids may well be important factors contributing to inhibitor specificity is provided by ATIII Denver, where the P'_1 residue Ser-394 is replaced by Leu, and which has lost antithrombin activity (Stephens et al., 1985).

In order to further examine the role of the P and P′ residues in serpin specificity, we have by in vitro site-directed deletion mutagenesis removed the P_1 residue (Arg-364) or the P'_1 residue (Met-365) of rα_2AP, thereby producing variant inhibitors with putative new reactive sites containing P_1Met-P'_1Ser and P_1Arg-P'_1Ser, respectively corresponding to the known reactive-site sequences of α_1AT or of ATIII. Deletion of the P'_1 Met actually results in displacing all of the P′ subsites by one position in the NH_2-terminal direction.

Analysis of rα_2AP-ΔMet365 reveals only the slightest change in activity toward plasmin and trypsin with predictably still no anti-elastase activity, but contrary to what was expected, there was no increase in antithrombin activity. In fact, a 20-fold decrease was observed. Apparently, α_2AP has no absolute requirement for Met in the P'_1 position in order to effectively inhibit plasmin or trypsin, as Ser is an adequate replacement. Natural P′ subsites also appear flexible. Thus, the primary inhibitory activity of α_2AP has not been abolished by a change in the P'_1 residue, contrary to what was observed in ATIII Denver. The fact that rα_2AP-ΔMet365 has not become an effective antithrombin may perhaps be related to the conspicuous absence of a proline residue in the P'_3 or P'_4 position, which occurs in ATIII and α_1AT Pittsburgh.

Analysis of rα_2AP-ΔArg364 showed that it is unreactive toward trypsin, plasmin, and thrombin but that it has acquired significant neutrophil elastase inhibitory capacity ($k_1 = 1.5 \times 10^5$ M^{-1} s^{-1}). This indicates that either the Met-362–Ser-363 or the Met-364–Ser-365 peptide bonds have now constituted a new reactive site. The removal of the bulky basic Arg-364 may have resulted in accessibility of these bonds for the active-site cleft of elastase.

In conclusion, it appears that α_2AP may serve as a valid alternative model to α_1AT for the detailed investigation of structure–function relationships in the reactive site of the serpins.

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