Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 4, pp 523–529, 1996
Published by Elsevier Science Ltd

0968-0896/96 $15.00 + 0.00

0968-0896(95)00379

Reversible Modification of Tissue-Type Plasminogen Activator by Methylphosphonate Esters†

Qinjian Zhao and Ildiko M. Kovach*
Department of Chemistry, The Catholic University of America, Washington, DC 20064, U.S.A.

Abstract—In spite of their rapid aqueous hydrolysis, 4-nitrophenyl 4-X-phenacyl methylphosphonates (X = H, (PMN) CH_3, CH_3O, Cl and NO_2) inactivate many serine proteases of the pancreatic and blood coagulation systems efficiently. The rate constants, k_i/K_i, for the inactivation of tissue-type plasminogen activator enzyme (t-PA) are 470–750 M^{-1} s^{-1} with PMN, 4-CH_3-PMN, and 4-CH_3O-PMN in pH 7.8, 0.05 M Tris buffer at 7.0 ± 0.5 °C, but t-PA cannot be inhibited with the 4-Cl and NO_2 derivatives due to rapid competing hydrolysis. Enzyme activity returns from each enzyme-adduct at a characteristic rate, due to a self-catalyzed intramolecular reactivation process. The rate constants for spontaneous reactivation of t-PA from the adducts formed with the three inhibitors are $k = 0.25–12.3 \times 10^{-2}$ min^{-1} at pH 7.4 and 25.0 ± 0.1 °C and pH-dependent with an apparent $pK \sim 8.3$. The recovery of t-PA activity from the adducts in 40% human plasma buffered at pH 7.4 is the same or twice that in plain buffer. The presence of fibrin has a slight effect on inactivation but not on reactivation. The modulation of enzyme activity by reversible generation of the phosphonylated adducts has potential for medical application. Published by Elsevier Science Ltd

Introduction

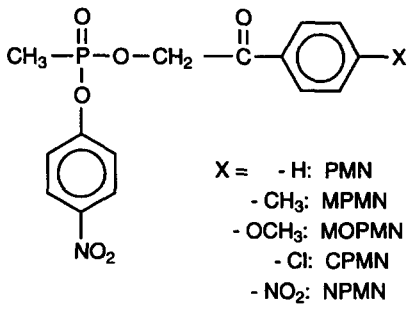
Recombinant tissue-type plasminogen activator (rt-PA)[1] enzyme is a new generation thrombolytic agent which is used in the treatment of acute myocardial infarction. The therapeutic properties of t-PA are superior, in some respects, to those of other thrombolytic agents, such as urokinase and streptokinase. The fibrinolytic process induced by t-PA is fibrin-specific, it causes only limited systemic plasminogen activation and fibrinogenolysis, and it does not induce an antibody response.[2–5] However, t-PA from human plasma is cleared rapidly with an initial half-life of 6 min.[2] Slowing down the clearance of t-PA from plasma would make its clinical administration more convenient. Consequently, the prolongation of t-PAs plasma half-life has attracted considerable interest and effort.[6] t-PA is eliminated almost exclusively by the liver via hepatocytes. Studies on the clearance through the hepatic receptor revealed that it preferentially binds t-PA complexes to plasminogen activator inactivator type I (PAI-1).[7–10] It was also reported that t-PA/PAI-1 complexes were eliminated twice as rapidly as free t-PA by isolated perfused rat liver.[11] Carbohydrate receptors are also important for the clearance of pharmacologic amounts of t-PA.[12–13]

It has been established that a free active-site serine residue, Ser478 in the catalytic triad Asp371-His322-Ser478,[14] is essential for the formation of stable complexes of t-PA with PAI-1.[15] When t-PA was inactivated with diisopropyl phosphofluoridate, the adduct did not react with PAI-1.[16,17] Thus covalent modification can provide protection against rapid inactivation of t-PA by PAI-1 ($k_i/K_i = 10^7$ M^{-1} s^{-1}) and retard the clearance of t-PA by hepatic cells. Modification of the catalytic Ser residue might be a welcome measure for medical applications if it is reversible. A number of thrombolytic enzymes have been modified by acylation, in which case the acyl enzyme undergoes slow deacylation to regenerate the free enzyme.[18–19] Reversible acylation of recombinant chimeric t-PA to provide control over the pharmacokinetic profile of the Smithkline and Beecham product has been recently reported.[20] However, most acyl-enzyme derivatives of t-PA are not stable enough for medical application and the acylation of t-PA by the modifiers can be slow.

Recent studies in this laboratory showed that serine proteases can be inactivated effectively with 4-nitrophenyl 4-X-phenacyl methylphosphonates (X = H, CH_3, CH_3O, Cl, NO_2, see acronyms and structures)[21,22] in spite of the rapid intramolecular hydrolysis of the compounds.[23] The phosphonylated enzyme adducts then undergo spontaneous dephosphonylation with anchimeric assistance from a carbonyl group in the side chain.[21,22] The advantages of phosphonylation over acylation are due to the extra valence in P compared to C. The possibility for inserting an extra ligand of the desired physical properties provides an additional leverage for diversity. Due to the similarity of the anionic alkylphosphonate monoester or alkylphosphonic acid products to physiologically important anionic phosphate esters, they are likely to be without adverse physiological properties. In fact, anionic electrophiles have recently been of interest for their

Key words: Fibrinolytic enzymes, serine protease, serine protease inhibition, t-PA inhibition, serine hydrolase inhibition.
†This research was supported by the American Heart Association, the Nation's Capital Affiliate.

potential antitumor activities. Here we report a *mechanistic study of the reversible modification of t-PA* by covalent attachment of the β-carbonyl-containing phosphonate fragment.



Results

Inactivation of t-PA

Measurements of the inactivation rate constants of t-PA by the PMN group of inhibitors is fraught with difficulties due to the rapid hydrolysis of the inhibitors and the limited analytical precision inherent in microtechniques. The protection of t-PA from aggregation also imposed constraints on the experimental conditions.[24] Optimal conditions were found at pH 7.8 and 7±0.5 °C. The loss of PMN, MPMN and MOPMN (see structures) due to hydrolysis was calculated to be less than 5% during incubation.[23] The rate constants for inactivation of two-chain t-PA by PMN, MPMN and MOPMN were calculated according to eq (1)[25]

$$1/\ln(v_o/v_i) = K_i/k_i t \cdot 1/[I] + 1/k_i t, \quad (1)$$

from four pairs of $\ln(v_i/v_o)$ activity vs. 1/[I] values, each measured at two fixed time increments (Figure 1). The method gives $K_i/k_i t$ (±SD) as the slope, the best defined parameter according to eq (1). The intercept value is generally subject to a large error and thus k_i and K_i are not well defined. The second-order rate constants for t-PA inactivation by PMN, MPMN and MOPMN were all in the range k_i/K_i = 470–750 M^{-1} s^{-1} and within ±25% relative SD for each case. From the intercept values, k_i values were in the range 0.003–0.05 s^{-1}, and from the ratio of slope and intercept values, K_i were calculated to be in the range 4–8 × 10^{-6} M.

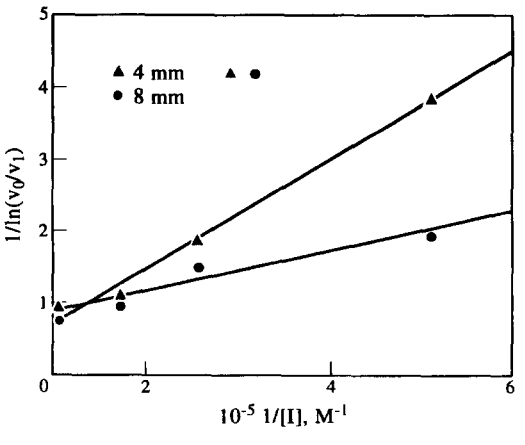


Figure 1. The inactivation of two-chain t-PA by MOPMN at pH 7.8 and 7.0±0.5 °C.

The kinetic rate constants for the inactivation of single-chain t-PA by the PMNs could not be measured with adequate precision because the reaction was too slow under these conditions and non-enzymic hydrolysis of the inhibitors outweighed t-PA inactivation at higher pH and temperature. Longer incubation times would have entailed excessive hydrolysis of the inhibitors as well as non-negligible enzyme recovery of activity, which would have invalidated the results. During the ~30 min incubation applied for maximal inactivation before the reactivation experiments, ca. 60% inactivation could be observed with single-chain t-PA, while ca. 90% inactivation of two-chain t-PA was achieved. The enzyme concentrations were based on activity assays that were performed systematically before and after inactivation in all cases. Therefore, the discrepancy reflects on the different rates of reaction of single-chain and two-chain t-PA with the PMNs rather than differential aggregation which would affect results of the enzyme activity assay as well. A qualitative observation was that the presence of fibrin had a small effect on the rates of inactivation of t-PA by PMN. Due to the rapid hydrolysis of CPMN and NPMN, there was no inhibition of t-PA activity observed after the incubation of two-chain t-PA with 1000-fold of CPMN or NPMN at a range of temperatures and pHs.

Reactivation of t-PA from the phosphonylated adducts

The rate constants for the reactivation of the adducts with the PMNs of single-chain t-PA, with and without fibrin present, and two-chain t-PA are very similar (Table 1). Complete recovery of t-PA (100±5%) was observed in all cases. This precludes the possibility of the incursion of any side reaction (aging) that would lead to irreversible inhibition of the enzyme.[22,26] A typical curve for the progress of reactivation is shown in Figure 2. The addition of BSA and triton X-100 to the reactivation buffer was necessary to stabilize the enzyme in solution in the lengthy experiments.

t-PA recovery from the adducts with PMN, MOPMN and MPMN was also studied in 40% human blood plasma buffered at pH 7.37 with 0.05 M phosphate buffer at 25.0±0.1 °C. The effect of human plasma on the recovery of the enzyme was negligible as compared to similar data in plain buffer at pH 7.37, shown in Table 2.

pH-Rate profiles for t-PA reactivation

The pH-dependence of the reactivation of two-chain t-PA from the adducts formed with PMN, MPMN and MOPMN is within 10-fold over 3.5 pH units. An ionizable group with a pK_a of 8.2–8.3 was observed from the pH-rate profiles (Fig. 3 and Table 3). These sigmoidal

Table 1. First-order rate constants for the reactivation of t-PA from its adducts with PMNs at 25.0 ± 0.5 °C

Compound	Reactivation buffer	k_{obs}, min^{-1} (SD)	$t_{1/2}$, min
Single-chain t-PA			
PMN	pH 7.50, 0.36 M Tris–HCl[a]	0.004 (0.003)[d]	173
		0.002 (0.001)[d]	346
	pH 8.23, 0.06 M Tris–HCl[b]	0.02 (0.05)[d]	30
	pH 8.23, 0.06 M Tris–HCl[a]	0.02 (0.03)[d]	36
MOPMN	pH 7.50, 0.36 M Tris–HCl[a]	0.06 (0.07)[d]	12
	pH 8.23, 0.06 M Tris–HCl[b]	0.04 (0.04)[d]	20
		0.05 (0.05)[c]	14
MPMN	pH 8.23, 0.06 M Tris–HCl[b]	0.03 (0.03)[c]	24
Single-chain t-PA with Fibrin			
PMN	pH 8.23, 0.06 M Tris–HCl[c]	0.04 (0.02)[c]	17
MOPMN	pH 8.23, 0.06 M Tris–HCl[c]	0.04 (0.01)[c]	20
MPMN	pH 8.23, 0.06 M Tris–HCl[c]	0.04 (0.01)[c]	16
Two-chain t-PA			
PMN	pH 8.23, 0.06 M Tris–HCl[c]	0.04 (0.01)[c]	27
MOPMN	pH 8.23, 0.06 M Tris–HCl[c]	0.028 (0.002)[e]	25
MPMN	pH 8.23, 0.06 M Tris–HCl[c]	0.04 (0.02)[e]	16

[a]No BSA and Triton X-100 in either reactivation buffer or assay buffer.
[b]0.5 mg/mL BSA and 0.1 g/L Triton X-100 in both reactivation buffer and assay buffer.
[c]Same as in b, except that the concentration of BSA is 1 mg/mL.
[d]The final concentration of S-2288 for activity assay is 0.83 mM.
[e]The final concentration of S-2288 for activity assay is 0.50 mM.

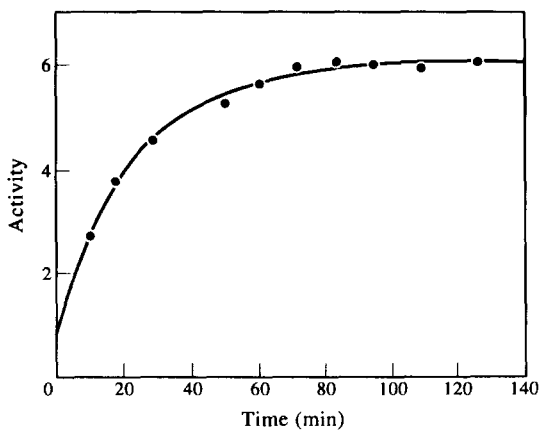


Figure 2. The reactivation of t-PA from its adduct with MOPMN at pH 9.07 and 25 ± 0.5 °C ($t_{1/2}$ = 16 min).

Table 2. First-order rate constants for the reactivation of two-chain t-PA from different adducts with PMNs in pH 7.37, 40% human plasma at 25 ± 0.5 °C

Compound	k_{obs}, min^{-1}	k_{obs},[a] min^{-1}
PMN	0.027 ± 0.004	0.025
	0.029 ± 0.010	
	0.033 ± 0.012	
MOPMN	0.033 ± 0.008	0.016
	0.040 ± 0.017	
MPMN	0.026 ± 0.020	0.018
	0.038 ± 0.022	

[a]Rate constant extrapolated from the pH-rate profile.

Table 3. The kinetic pK and pH-independent rate constants for the reactivation of two-chain t-PA from different adducts with PMNs

Compound	$10^3 k_{lim1}$, min^{-1}	$10^3 k_{lim2}$, min^{-1}	pK
PMN	18 ± 2	72 ± 4	8.2 ± 0.1
MOPMN	12.2 ± 0.5	48.9 ± 0.9	8.4 ± 0.1
MPMN	2.5 ± 1.1	123 ± 11	8.2 ± 0.1

curves for the reaction with PMNs were calculated using the non-linear least squares fit of k_{obs} values to the eq (2) below.

$$k_{obs} = \frac{k_{lim1} K_a}{K_a + [H^+]} + k_{lim2}. \quad (2)$$

Discussion

Inactivation of t-PA

The two-chain form of t-PA with its well developed catalytic apparatus can react effectively with three of the five PMN derivatives studied. This mature form seems to compete better than the single-chain form with the rapid aqueous hydrolysis of these inhibitors,[22,23] thus the inactivation is more efficient. The observation is consistent with earlier reports on much higher thrombolytic activity[2,27] and amidolytic activity, even with the small chromogenic substrate S-2288,[24] of two-chain t-PA than its single-chain form.

The second-order rate constants at 25.0 °C for t-PA inactivation are smaller than those for the inactivation of trypsin by PMN, 884 M^{-1} s^{-1}, and much smaller than those for the inactivation of α-chymotrypsin, between 37,000 and 770,000 M^{-1} s^{-1}, with the same compound.[22] This might be expected, since the two aromatic rings make the inhibitors very hydrophobic, which complies with the specificity of chymotrypsin for susbtrates. The blood cascade enzymes,[28] t-PA and trypsin all have the specificity for cleaving a peptide bond with an Arg or Lys at the carboxyl side. In general, there are more stringent specificity requirements in the blood cascade enzymes and t-PA than in trypsin.[29]

Reasonably strong binding of the PMNs to t-PA is associated with constants in the μM range. The k_i values probably measure the rate of P—O bond formation, since departure of the 4-nitrophenyl leaving group might be expected to be fast.[30] Although precise values of k_i have not been reported yet for phosphonylation of the active site Ser in serine hydrolases, our earlier results with acetylcholinesterase phosphonylation by 4-nitrophenyl esters of alkylphosphonates gave values 10- to 50-times greater at 25 °C than the k_i values for phosphonylation of t-PA.[25]

Enantioselectivity

A very significant enantioselectivity of the inactivation of, particularly, chymotrypsin (90–1900) and trypsin

has been observed and reported recently.[22] Only a minimum enantioselectivity between 5 and 49 could be had for the inactivation of trypsin by the PMNs, since hydrolysis of the (+) enantiomer prevents enzyme inactivation. The reaction of t-PA with the PMNs was much more difficult to monitor than reactions of chymotrypsin and trypsin. The resolved (−) enantiomer of MPMN inactivated t-PA at similar rates to the racemate but attempts to inactivate t-PA with the resolved (+) enantiomer of MPMN failed under these conditions. This indicates a minimum enantioselectivity of 10.

In the serine proteases, two interactions govern the mode of binding: the hydrophobic interaction in the specificity pocket and the electrostatic interaction between P=O and the hydrogen donors of the oxyanion hole.[26,31-39] Our earlier molecular models of minimum-energy structures of trypsin adducts indicated that these interactions should become very strong for the P_S enantiomer and thus enforce an in-line displacement at P by the attacking Ser hydroxyl.[22] It has also been shown that the faster reacting, levorotatory, enantiomer has the P_S absolute configuration in analogous phosphonate esters.[40,41]

Both electronic and steric effects of the *para* substituents in the phenacyl group affect the enantioselectivity of trypsin toward PMNs. Strong interactions exist between the phenacyl moiety and the specificity pocket at the active site according to the molecular mechanics calculations. These calculations also showed a possible hydrogen bonding interaction of the carbonyl with a peptide N—H.[42] It is very likely that the interactions of the PMNs at the active site of t-PA are similar to those described above.

Reactivation of t-PA from the adducts with the PMNs

Once the adducts of t-PA were formed with the PMNs, the recovery of t-PA activity showed the same characteristics regardless of the form of the enzyme. The recovery rates of single chain t-PA may have been slightly slower from the adducts with PMN, but the difference is within experimental error. The presence of fibrin made the inactivation of single-chain t-PA easier, while it had no effect on the rate of recovery of t-PA activity (Table 1). This is consistent with the fact that the binding site of fibrin to single-chain t-PA is not at or near the active site of t-PA, even though the presence of fibrin enhances t-PA by affecting its global structure, which is the origin of the high fibrin-specificity of t-PA in thrombolysis.[43]

The pH-dependence of t-PA recovery, shown in Figure 3, was nearly identical to that observed when trypsin was inhibited with the three compounds.[22] We suggest the participation of an active-site residue as a general base in the catalysis of hydration of the carbonyl and the related protonic equilibrium in the course of enzyme reactivation, as shown in Scheme 1.[44] The pH dependence has been the strongest for the reactivation

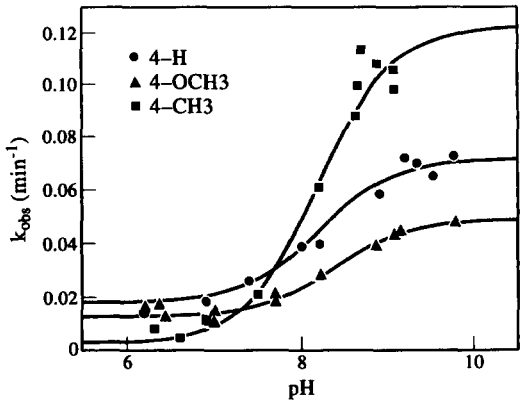
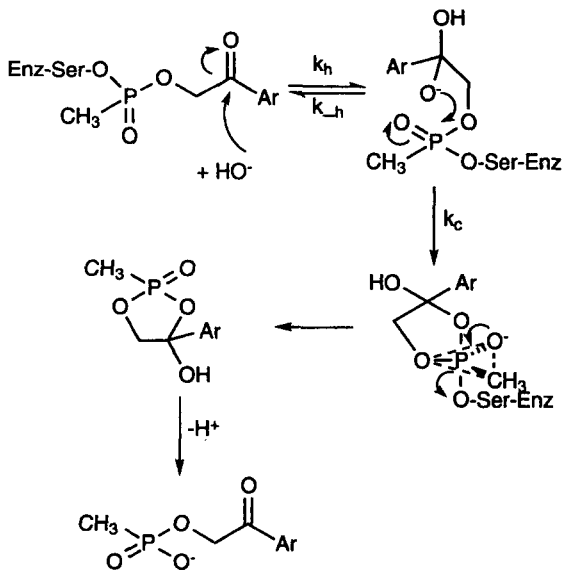


Figure 3. pH-Rate profiles for the reactivation of two-chain t-PA from its adducts with 4-substituted PMNs at 25 ± 0.5 °C.

of t-PA from the MPMN adduct. This may imply that the orientation of the carbonyl in this adduct is more favorable for interaction of the molecule with the basic residue at the active site. It would be tempting to propose that the active site His is involved in this proton transfer, except that the His residue was seen to remain protonated (acidic form) after the phosphonylation step.[26] It also seems possible that the local conformation of the active site in the adducts is dependent on the phosphonyl fragment. If so, the His may transfer the proton to adjacent solvate water or other residues and resume its base catalytic function in some cases, such as the MPMN-adduct, but remain unavailable for catalysis in other cases.

Mechanism of reactivation of t-PA

Results of this and previous work corroborate a common mechanism of reversible inhibition of serine proteases via intramolecular dephosphonylation. The nature of the pH dependence and small solvent isotope effects, ~1.2[21] for trypsin and chymotrypsin, support a



Scheme 1.

mechanism in which proton transfer is not rate determining: rapid hydration of the phenacyl group with or without general-base catalysis by an active-site general base is followed by an intramolecular attack of the carbonyl hydrate anion at the central phosphorus atom and by removal of the enzymic Ser residue. Scheme 1 shows details of the reaction and eq (3) gives the rate law using the steady state approximation.

$$k_{obs} = \frac{k_h' k_c}{k_{-h} + k_c} \quad \text{and} \quad k_c = \frac{k_{obs} k_{-h}}{k_h' - k_{obs}} \tag{3}$$

The rate-determining step has been shown, in our previous work, to be the hydration of the carbonyl in the mechanism of the non-enzymic hydrolysis of MPMN.[23] The exchange of ^{18}O from the medium (50%) is consistent with the contention that the rate constant for cyclization (k_c) is greater than that for dehydration (k_{-h}). This latter was estimated from the appropriate elementary rate constants and equilibria to be $\sim 10^6$ s^{-1}. Moreover, the value of k_{-h} is not expected to be sensitive to the differences in leaving group between MPMN and the corresponding Ser ester in the enzyme adduct. The value of $k_{h'}$ is available for MPMN, from previous work, under a wide range of conditions. Thus the rate constant for cyclization (k_c) can be calculated from eq (3) for the reactivation of t-PA from the 4-methylphenacyl methylphosphonate adducts.

The calculation yields $k_c \sim 10^5$ s^{-1}. In comparison, $k_c = 3.4 \times 10^4$ s^{-1} for the reactivation of trypsin, 1.2×10^5 s^{-1} for the reactivation of α-chymotrypsin from the adduct formed with the (+) enantiomer, and $4.5 \times 10^3 s^{-1}$ for the reactivation of α-chymotrypsin from the adduct with the (−) enantiomer of MPMN. This result confirms that the cyclization step is 10 times slower than reversion of the carbonyl hydrate to starting materials.

The rate of reactivation of t-PA is then limited either by P—O bond breaking to Ser or by concerted displacement at P via cyclization. This conclusion holds broad validity for serine protease reactivation from the adducts with the PMN group of inhibitors, except for the fastest reactivation of α-chymotrypsin from the adducts with MPMN and the (+) enantiomers of PMNs.

The broad range of rates of serine protease reactivation is consistent with self-catalyzed enzyme reactivation from the adducts with variant efficiency characteristic for the individual phenacyl substituent interaction with the binding site (specificity pocket or other residues including solvate water).

The rate constants for t-PA reactivation from its adducts with PMN, MPMN and MOPMN in 40% human plasma are listed in Table 2. The similarity of rate constants in aqueous buffer solution and human plasma solution suggests that components in the plasma do not affect the reactivation of t-PA. Only the enzyme itself and pH play an important roles in the dephosphonylation of the active-site serine residue in the adducts of t-PA formed with phosphonate inhibitors.

Conclusions

The two-chain form of t-PA can be inhibited efficiently and reversibly with 4-H, 4-CH_3, and 4-OCH_3-PMNs. The covalently modified t-PA adducts seem to have the propensity to release active t-PA under physiological conditions. A rational selection of the substituents in the 4-position of phenacyl can provide inhibitors that will inactivate t-PA. Enzyme activity may be regained on different time scales depending on the electronic and structural features of the modifier. Implications for biomedical use of these compounds as temporary modifiers of enzyme activity through controllable inactivation–reactivation cycles is a definite benefit of this investigation.

Experimental

Instrumentation

Conventional kinetic measurements were carried out with a Perkin–Elmer L-7 UV/Vis spectrophotometer interfaced to a Zenith Z-100 computer. The sample chamber of the instrument was furnished with circulating water provided by an MGW Lauda RMS-20 water bath. The temperature was monitored by a digital readout with a thermistor probe. All kinetic data were analyzed with the OLIS or GraFit kinetics software.[45]

The pH was measured with a Radiometer PHM84 Research pH meter furnished with a Fisher combination microprobe electrode. An SLM-Aminco SPF-500C spectrofluorometer was used for spectrofluorometric measurements for the titration of serine protease activity. Separations of enantiomers of phosphonates were carried out with a Waters (Milford, MA) HPLC instrument and a Chiralcel-OJ (Daicel Industries, Ltd) column with cellulose-based chiral stationary phase. The output signal from a Lambda-Max-481 detector was digitized by interfacing to an IBM-XT computer through a data acquisition board (DAS-8PGA, Keithley Metrabyte). The optical rotation was measured with an AutoPol-II polarimeter (Rudolph Research, NJ).

Materials

Single-chain t-PA (EC 3.4.21.68, 95% 63 kDa with some 65 kDa forms) from human cell culture was purchased from CalBiochem. Human fibrin monomer and the chromogenic substrate, H-D-Ile-Pro-Arg-pNA (S-2288), were purchased from KabiVitrum Inc. Triton X-100, human plasmin, bovine lung aprotinin, bovine serum albumin (BSA), *N*-glutaryl-Gly-Arg-7-amido-4-methylcoumarin and buffer salts were Sigma products. The synthesis and purification of the inhibitors was described earlier.[23,46] They were >98% pure

and the stock solutions were analyzed frequently for intact ester by hydrolyzing it and calculating the stoichiometric release of 4-nitrophenol.

Solutions

The t-PA stock solution was 7.7×10^{-6} M in water containing 0.1 g L^{-1} Triton X-100. S-2288 was dissolved in 0.0001 N HCl into a 10 mM stock solution. pH 8.23, 0.06 M Tris–HCl buffer with or without 0.5 mg mL^{-1} BSA, 0.15 M NaCl and 0.1 g L^{-1} Triton X-100 was used for t-PA activity assay.

Enzyme activity assay

The reactivation and activity assays were carried out at 25.0 ± 0.5 °C. A 100 μL aliquot of the reaction mixture and 100 μL S-2288 stock solution were introduced to 1000 μL assay buffer after it had been equilibrated for 15 min. The release of *p*-nitroaniline was monitored at 405 nm by a Perkin–Elmer Lambda-7 UV/Vis spectrophotometer. The initial rate of the substrate hydrolysis reflects t-PA activity.

Reversible inhibition of single-chain t-PA

Inactivation. To 30 μL 7.7×10^{-6} M t-PA, 10 μL pH 7.5, 0.72 M Tris–HCl buffer was introduced. After mixing, the reaction mixture was incubated in an ice-water bath for 5 min. About 300-fold excess of inhibitor, over t-PA, in 20 μL anhydrous methanol was introduced. The mixture was shaken thoroughly and allowed to stay in the ice-water bath for 15–20 min.

Reactivation of single-chain t-PA from its adducts. 600 μL buffer for reactivation, as given in Table 1, was introduced to the above-mentioned mixture to initiate reactivation of t-PA and the stopwatch was started at the same time. Control experiments were carried out as described above, except that 20 μL methanol was added instead of the inhibitor solution. No loss of t-PA activity was observed due to inhibition by methanol. 1 mg mL^{-1} BSA and 0.1 g L^{-1} Triton X-100 were included in the reactivation buffer to provide constant t-PA activity in the course of the reactivation experiments.

First-order rate constants of t-PA reactivation were obtained with their standard deviation from a non-linear least squares fit of t-PA activity (the initial rate) vs. time to the first-order rate law.

Reversible inhibition of two-chain t-PA

Preparation of two-chain t-PA.[27] Single-chain t-PA (100 μL, 1.5×10^{-6} M) and human plasmin (50 μL of a 0.88 casein units mL^{-1} solution in 0.04 M, pH 7.8 phosphate buffer) were mixed well and incubated at rt for 90 min. To the above solution, aprotinin (40 μL of 10 trypsin inhibitory units mL^{-1} solution) was introduced and the mixture was incubated at rt for 15 min.

Inhibition and reactivation of two-chain t-PA. The experimental procedures were very similar to the procedures described above for single-chain t-PA, except that the final concentration of t-PA during the inhibition process was 7×10^{-7} M and the incubation period for inactivation was 30 min. Reactivation experiments were also performed in buffers containing 40% human plasma. The human plasma was prepared from fresh whole blood of a volunteer after removing the cells by centrifugation.

Kinetics of inactivation of two-chain t-PA. The inactivation of two-chain t-PA by 4-H, 4-CH_3, and 4-OCH_3 PMN was studied with a fluorogenic substrate, *N*-glutaryl-Gly-Arg-7-amido-4-methylcoumarin. 120 μL two-chain t-PA solution was pre-equilibrated in pH 7.8, 0.05 M Tris buffer at 7 °C for 15 min followed by mixing with 5–25 μL dilute solutions of the PMNs in methanol. The final inhibitor concentration in the reaction mixtures was between 1.8 and 8.8×10^{-6} M and the concentration of two-chain t-PA was much lower. The reaction mixtures were incubated at 7 °C and the activity assays were taken at 4, 8 and 12 min from the addition of inhibitor. The consumption of the substrate during the activity assay using initial rates was within 0.4%. A series of residual enzyme activity was obtained after a fixed incubation time with different inhibitor concentrations. The second-order rate constants for the inactivation of t-PA by the inhibitors were then calculated according to eq (1).

Linear least squares fit of 4 $\ln(v_i/v_o)$ activity vs $1/[I]$ pairs gave the k_i/K_i values with standard deviation from 1/slope $\times t$ for at least two values of t. Values of k_i with their standard deviation can be calculated from the inverse of the intercept value, which is poorly defined. A typical example is shown in Figure 1.

References and Notes

1. Abbreviations: BSA, bovine serum albumin; Chiralcel-OJ, cellulose tri-4-methyl-benzoate coated on silica; EDTA, ethylene diamine tetraacetate; PMNs, 4-nitrophenyl phenacyl methylphosphonates; OP, organophosphorus; MPMN, 4-nitrophenyl, 4-methylphenacyl methylphoshponate; MOPMN, 4-nitrophenyl 4-methoxyphenacyl methylphosphonate; CPMN, 4-nitrophenyl 4-chlorophenacyl methyl phosphonate; NPMN, 4-nitrophenyl 4-nitrophenacyl methylphosphonate; *p*NA, *p*-nitroanilide; HPLC, high pressure liquid chromatography; Tris, tris hydroxymethyl aminoethane; t-PA, tissue-type plasminogen activator enzyme; S-2288, H-D-Ile-Pro-Arg-*p*NA.

2. Collen, D.; Lijmen, H. R.; Todd, P. A.; Goa, K. L. *Drugs* **1989**, *38*, 346.

3. Collen, D.; Lijen, H. R. *Biochem. Pharmacol.* **1990**, *40*, 177.

4. Van Zonneveld, A. J.; Vries, C. D.; Pannekoek, H. In *Serine Proteases and Their Serpin Inhibitors in the Nervous System*; Festoff, B. W., Ed.; Plenum: New York, 1990; pp 51–68.

5. Verstraete, M.; Collen, D. *Blood* **1986**, *67*, 1529.

6. Tanswell, P.; Seifried, E.; Stang, E.; Krause, J. *Drug Res.* **1991**, *41(II)*, 1310.

7. Owensby, D. A.; Morton, P. A.; Schwartz, A. L. *J. Biol. Chem.* **1989**, *264*, 18180.

8. Morton, P. A.; Owensby, D. A.; Wun, T. C.; Billadello, J. J.: Schwartz, A. L. *J. Biol. Chem.* **1990**, *265*, 14,099.

9. Wing, L. R.; Bennett, B.; Booth, N. A. *FEBS Lett.* **1991**, *278*, 95.

10. Fuchs, H. E.; Berger, Jr. H.; Pizzo, S. V. *Blood* **1985**, *65*, 539.

11. Wing, L. R.; Hawksworth, G. M.; Bennett, B.; Booth, N. *J. Lab. Clin. Med.* **1991**, *117*, 109.

12. Cole, E. S.; Nichols, E. H.; Poisson, L.; Harnois, M. L.; Livingston, D. J. *Fibrinolysis* **1993**, *7*, 15.

13. Hajjar, K. A.; Reynolds, C. M. *J. Clin. Invest.* **1994**, *93*, 703.

14. Pennica, D.; Holmes, W. E.; Kohr, W. J.; Harkins, R. N.; Vehar, G. A. *Nature* **1983**, *301*, 214.

15. Monge, J. C.; Lucore, C. L.; Fry, E. T. A.; Sobel, B. E.; Biladello, J. J. *J. Biol. Chem.* **1989**, *264*, 10,922.

16. Chmielewska, J.; Ranby, M.; Wiman, B. *Biochem. J.* **1988**, *251*, 327.

17. Thorsen, S.; Philips, M.; Selmer, J.; Lecander, I.; Asted, B. *Eur. J. Biochem.* **1988**, *173*, 33.

18. (a) Sturzebecher, J.; Richter, M.; Markwardt, F. *Thromb. Res.* **1987**, *47*, 699. (b) Sturzebecher, J. *Biomed. Biochem. Acta* 1986, *45*, 1405.

19. Kalindjian, S. B.; Smith, R. A. G. *Biochem. J.* **1987**, *248*, 409.

20. Wilson, S.; Cronk, D. W.; Dodd, A. F.; Esmail, S. B.; Kalindian, L.; McMurdo, L.; Browne, M. J.; Smith, R. A.; Robinson, J. H. *Thromb. Hemostas.* **1993**, *70*, 984.

21. Kovach, I. M.; McKay, L. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1735.

22. Zhao, Q.; Kovach, I. M.; Bencsura, A.; Papathanassiu, A. *Biochemistry* **1994**, *33*, 8128.

23. Kovach, I. M.; Zhao, Q.; Keane, M.; Reyes, R. *J. Am. Chem. Soc.* **1993**, *115*, 10471.

24. Husain, S. S. *Arch. Biochem. Biophys.* **1991**, *285*, 373.

25. (a) Kovach, I. M. *J. Enzyme Inhib.* **1991**, *4*, 201. (b) Bennet, A. J.; Kovach, I. M.; Bibbs, J. *J. Am. Chem. Soc.* 1989, *111*, 6424. (c) Bennet, A. J.; Kovach, I. M.; Schowen, R. L. *J. Am. Chem. Soc.* 1988, *110*, 7892.

26. (a) Kovach, I. M. *J. Enzyme Inhib.* 1988, *2*, 199. (b) Kovach, I. M. *J. Mol. Struct. Theochem.* **1988**, *76*, 159.

27. Bennett, W. F.; Paoni, N. F.; Keyt, B. A.; Botstein, D.; Jones, A. J. S.; Presta, L.; Wurm, F. M.; Zoller, M. J. *J. Biol. Chem.* **1991**, *266*, 5191.

28. Davie, E. W.; Fujikawa, K.; Kisiel, W. *Biochemistry* **1991**, *30*, 10,363.

29. Lottenberg, R.; Christensen, U.; Jackson, C. M.; Coleman, P. L. *Meth. Enzymol.* **1981**, *80*, 341.

30. Kovach, I. M.; Bennet, A. J.; Bibbs, J. A.; Zhao, Q. *J. Am. Chem. Soc.* **1993**, *115*, 5138.

31. Kovach, I. M.; Huber-Ashley, H. J.; Schowen, R. L. *J. Am. Chem. Soc.* 1988, *110*, 590.

32. Kovach, I. M.; McKay, L.; Vander Velde, D. *Chirality* **1993**, 143.

33. Stroud, R. M.; Kay, L. M.; Dickerson, R. E. *J. Mol. Biol.* **1974**, *83*, 185.

34. Kossiakoff, A. A.; Spencer, S. A. *Biochemistry* **1981**, *20*, 6462.

35. Grunwald, J.; Segall, Y.; Shirin, E.; Waysbort, D.; Steinberg, N.; Silman, I.; Ashani, Y. *Biochem. Pharmacol.* **1989**, *38*, 3157.

36. Gorenstein, D. G.; Shah, D.; Chen, R.; Kallick, D. *Biochemistry* **1989**, *28*, 2050.

37. Adebodun, F.; Jordan, F. *J. Cell. Biochem.* **1989**, *40*, 249.

38. Markley, J. L. In *Biological Applications of Magnetic Resonance*; Shulman, R. G., Ed.; Academic: New York, 1979; p 397.

39. Van der Drift, A. C. M.; Beck, H. C.; Dekker, W. H.; Hulst, A. G.; Wils, E. R. *Biochemistry* **1985**, *24*, 6894.

40. de Jong, L. P. A.; Benschop, H. P. In *Stereoselectivity of Pesticides*; Ariens, E. J.; van Rensen, J. J. S.; Welling, W., Eds.; Elsevier: Amsterdam, 1988; pp 109–149.

41. Note that in-line displacement of 4-nitrophenol by serine causes no change in notation of the configuration around P because 4-nitrophenol has the highest priority in the reactant, but serine is only second in priority in the adduct.

42. Bencsura, A., personal communication.

43. de Vos, A. M.; Ultsch, M. H.; Kelley, R. F.; Padmanabhan, K.; Tulinsky, A.; Westbrook, M. L.; Kossiakoff, A. A. *Biochemistry* **1992**, *31*, 270.

44. Covalent modification of Ser by the carbonyl group of the molecules is discounted based on (1) the facility and character of the reactivation of t-PA, (2) the results of the ^{31}P NMR of the adduct of chymotrypsin[22] and (3) by the identity of the rates of 4-nitrophenol release and loss of enzyme activity. Addition of the Ser nucleophile to carbonyl followed by an instantaneous rearrangement to the phosphonylated enzyme derivative seems highly unlikely as inferred from molecular models.

45. Leatherbarrow, R. J. GraFit Version 3.0, Erithacus Software Ltd.: Staines, U.K., 1992.

46. Lieske, C. N.; Hovanec, J. W.; Steinberg, G. M.; Pikulin, J. N.; Lennox, W. J.; Ash, A. B.; Blumbergs, P. *J. Agric. Food Chem.* **1969**, *17*, 255.

(*Received in U.S.A.* 15 *September* 1995; *accepted* 7 *November* 1995)