An active-site titrant for human tissue-type plasminogen activator

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The reaction of recombinant tissue-type plasminogen activator with the inverse substrate 4-amidino-2-nitrophenyl 4'-anisate results in the rapid release of the chromogen 4-amidino-2-nitrophenol and the accumulation of the relatively stable 4-anisoyl-enzyme. Spectrophotometric monitoring of the reaction enables the operational molarity of the enzyme to be determined.

INTRODUCTION

Human tissue-type plasminogen activator (t-PA) has attracted much attention as a potential thrombolytic agent for the treatment of acute myocardial infarction and other thrombotic disorders. Activation of plasminogen by t-PA has been shown to be stimulated by fibrin (Hoylaerts *et al.*, 1982). In animal studies of thrombolysis, that property has been reflected in a higher degree of clot selectivity and a lesser extent of systemic activation of fibrinolysis than that exhibited by established thrombolytic agents (Matsuo *et al.*, 1981; Korninger *et al.*, 1982; Collen *et al.*, 1983). The availability of significant amounts of enzyme through expression of the cloned t-PA gene (Pennica *et al.*, 1983) has permitted clinical evaluation of r-tPA (Van de Werf *et al.*, 1984; Verstraete *et al.*, 1985). Active-site titrants have been widely used for the quantification of functional catalytic centres in serine proteinases, particularly those with trypsin-like specificity. The most commonly used such substrates are esters or thioesters of *p*-guanidinobenzoic acid (Chase & Shaw, 1967, 1969; Melhado *et al.*, 1982; Cook & Powers, 1983). Studies in this laboratory (Smith *et al.*, 1981, 1982; Dupe *et al.*, 1984, 1985) have shown that plasminogen activators may be utilized as thrombolytic agents in the form of acyl-enzymes whose stability may be manipulated chemically in order to achieve control over the clot selectivity and pharmacodynamics of thrombolysis. For a comparison of acyl derivatives of different fibrinolytic enzymes on a molar basis, we required a simple chromogenic active-site titrant of t-PA. I show here that *p*-nitrophenyl *p*'-guanidinobenzoate (NpGB) is unsuitable for this purpose and that a new substrate based on an 'inverse' ester structure (Tanizawa *et al.*, 1977) fulfils many of the requirements of a useful titrant of t-PA.

EXPERIMENTAL

Materials

p-Aminodiphenol hydrochloride and APAN hydrochloride were prepared as described previously (Smith *et al.*, 1982; Tanizawa *et al.*, 1977). Phe-Pro-Arg-CH₂Cl was obtained from Universal Biologicals, Cambridge, U.K. 4-amidino-2-nitrophenol hydrochloride was prepared by addition of *p*-aminodiphenol hydrochloride

(32.5 mmol) to concentrated HNO₃ (relative density, *d*, 1.42; 15 ml) at 0 °C. The mixture was stirred and allowed to warm to ambient temperature over 16 h. The product was isolated by dilution into saturated NaCl solution (50 ml), filtration and recrystallization [from propan-2-ol/water (1:1, v/v) then ethanol/water (10:1, v/v)] as yellow crystals [m.p. 190 °C (decomp.); yield: 1.76 g (25%)]. The p.m.r. spectrum of the material was consistent with the proposed structure. APNAN was prepared by reaction of the above phenol (5 mmol) with *p*-anisoyl chloride (5.5 mmol) in dry pyridine (10 ml) at ambient temperature for 30 min. The product was isolated in 14% final yield by evaporation, precipitation from propan-2-ol solution with diethyl ether and recrystallization (twice) from propan-2-ol/ethyl acetate (1:1, v/v) [white amorphous solid, m.p. 162 °C; p.m.r. (2H₂O): δ 9.8 p.p.m., doublet (four protons) exchangeable in 2H₂O; δ 9.0 p.p.m., doublet (one proton); δ 8.4/8.7 p.p.m., doublet of doublets (one proton); δ 7.2/8.2 p.p.m., quartet (four protons); δ 7.9 p.p.m., doublet (one proton); δ 3.9 p.p.m., singlet (three protons)]. NpGB was obtained from Sigma Chemical Co., Poole, Dorset, U.K. Acylating agents were stored as stock solutions of concentration of 10 mM in dry DMSO at -40 °C and diluted to appropriate working concentrations immediately before use. Substrate S-2288 was obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. r-tPA was purified to homogeneity from the culture supernatant of genetically manipulated Bowes-melanoma cells (Browne *et al.*, 1985). The product had *M_r* 63000–65000 by SDS/polyacrylamide-gel electrophoresis combined with fibrin-overlay zymography (Granelli-Piperno & Reich, 1978) and was isolated predominantly in the single-chain form. It was stored at a concentration of 2–3 mg/ml in 0.1 M-sodium 4-guanidinobutyrate/0.3 M NaCl/0.02 M-sodium phosphate/0.01% Tween 80, pH 7.4, at -40 °C.

Methods

Active-site titration. r-tPA (25–200 μl of stock solution) was mixed with 0.5 M-L-arginine hydrochloride/0.5 M-NaCl/20 mM-Tris/HCl/0.01% Tween 80, pH 7.4 (ANT buffer) to a final volume of 0.5 ml. APNAN (50 μl of a 500 μM solution in DMSO) was added and the increase in *A*₄₀₅ monitored in a single-beam recording spectro-

Abbreviations used: t-PA, tissue-type plasminogen activator; r-tPA, recombinant tissue-type plasminogen activator produced in Bowes-melanoma cells; APNAN, 4-amidino-2-nitrophenyl 4'-anisate; NpGB, *p*-nitrophenyl *p*'-guanidinobenzoate; APAN, *p*-aminodiphenol *p*-anisate; Phe-Pro-Arg-CH₂Cl, phenylalanylprolylarginine chloromethane ('chloromethyl ketone'); DMSO, dimethyl sulphoxide; S-2288, H-D-isoleucylprolyl-arginine *p*-nitroanilide dihydrochloride.

photometer (Pye-Unicam SP.6-350) over 3–4 min at 25 °C. The burst absorbance was estimated by extrapolation of the linear post-burst increase in A_{405} to zero time. Plots of A_{405} against sample volume were extrapolated to undiluted stock solution by linear-regression analysis. A value of $\epsilon_{405} = 5028 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for 4-amidino-2-nitrophenol under these conditions was used to calculate the molarity of the test r-tPA solution.

Deacylation-rate-constant determination. r-tPA (0.2 ml of a 43 μM stock solution) was treated with an equimolar amount of APNAN at 0 °C for 30 min. The acyl-enzyme was diluted 1:100 in ANT buffer and warmed to 25 °C. Regeneration of amidolytic activity at this temperature was measured by using 1.0 mM-S-2288 in 0.1 M-triethanolamine/HCl, pH 8.0 (0.5 ml) at 25 °C. Release of *p*-nitroaniline was monitored spectrophotometrically at 405 nm and the pseudo-first-order rate constant calculated as described previously (Chase & Shaw, 1969).

Acylation-rate-constant determination. r-tPA (0.1 μM in ANT buffer, 2.0 ml) was mixed with stock solution (50 μM in DMSO) to give a final concentration of 0.4–2.0 μM and incubated at 25 °C. The activity of the solution was determined at 1 min intervals for 4–10 min by assay with S-2288 as described above. Apparent first-order inactivation rate constants (k_{in}) and the derived second-order rate constant (k_2/K_s) were obtained by application of the kinetic analysis described by Bender *et al.* (1967) and Chase & Shaw (1969).

RESULTS AND DISCUSSION

When r-tPA was mixed with an excess of NpGB in a buffer containing L-arginine (used to ensure the solubility of the enzyme), a slow increase in A_{405} was observed (Fig.

1a). This increase could be blocked by the affinity label Phe-Pro-Arg-CH₂Cl, which has been shown to be a rapid and irreversible inhibitor of t-PA (Korninger *et al.*, 1981). Complete reaction of NpGB with r-tPA under these conditions required 2 h and resulted in overall turnover of substrate. Turnover of methylumbelliferyl *p*-guanidinobenzoate by porcine r-tPA has been noted elsewhere (Walker, 1982). When t-PA was incubated with NpGB in equimolar proportions, very little ($\leq 20\%$) inactivation of the enzyme occurred under conditions (see under 'Methods') where APNAN caused essentially complete abolition of the amidolytic activity of the enzyme. Fig. 1(a) shows that APNAN produced a burst of A_{405} characteristic of rapid enzyme acylation and accumulation of acyl-enzyme. In this case the post-burst absorbance increase was not completely blocked by the tripeptide chloromethane, suggesting that non-specific hydrolysis rather than substrate turnover was occurring. If the increase in A_{405} was corrected for such hydrolysis by extrapolation to zero time, a plot of the burst absorbance against volume of test r-tPA solution was found to be linear (Fig. 1b). Non-zero absorbance in the absence of added r-tPA was caused by small variable amounts of chromogen generated by hydrolysis of APNAN in the reagent stock solutions. Prior reaction of r-tPA with the non-chromogenic substrate APAN [which has been shown to acylate other enzymes specifically (Tanizawa *et al.*, 1977)] blocked the concentration-dependent reaction with APNAN (Fig. 1b). Thus both inverse substrates appear to react at the same site. In order to calculate the operational molarity of r-tPA from the slope of a plot such as that of Fig. 1(b), it is necessary to show that the rate constant for acyl-enzyme formation (k_2 ; Bender *et al.*, 1967) is much greater than that for acyl-enzyme hydrolysis (k_3). The latter was found to be $1.2 \times 10^{-4} \text{ s}^{-1}$ (see under 'Methods'). Fig. 2 shows

Fig. 1. Reaction of APNAN with r-tPA

(a) Release of chromogens from r-tPA (8 μM on ANT buffer) at 25 °C on addition of 45 μM -APNAN (upper trace) or 45 μM -NpGB (lower trace). Arrows indicate the point of addition of Phe-Pro-Arg-CH₂Cl to a final concentration of 0.87 mM. (b) Dependence of 'burst' A_{405} on amount of r-tPA in a typical titration. ●, unmodified r-tPA, ▲, r-tPA with the active centre blocked by preincubation with APAN (200 μM , 25 °C, 30 min in ANT buffer).

Fig. 2. Determination of the second-order rate constant (k_2/K_s) for the reaction of APNAN with r-tPA

Mean values ($\pm$ S.E.M.) for four determinations and three different batches of (predominantly two-chain) r-tPA are shown.

a double-reciprocal relationship between the apparent first-order rate constant for inactivation of r-tPA by excess APNAN (k_{in}) and the concentration of the titrant. The slope of that line gave the second-order inactivation rate constant, $k_2/K_s = 6300 \text{ M}^{-1} \cdot \text{s}^{-1}$, where K_s is the substrate dissociation constant. Titration theory equates the value of k_{in}^{-1} at infinite [APNAN] with k_2^{-1} (Bender *et al.*, 1967), but the present data do not permit accurate individual determinations of k_2 and K_s by extrapolation. However, inspection of Fig. 2 shows that k_2 is probably $> 0.1 \text{ s}^{-1}$, which is about 1000-fold higher than k_3 . APNAN therefore meets the main kinetic condition for an active-site titrant.

APNAN has been used for the routine determination of active centres in purified r-tPA batches prepared in this laboratory. By using fibrin-plate and clot-lysis-time assays calibrated with the recently established international t-PA standard (Gaffney & Curtis, 1985), it was found that the specific fibrinolytic activity of such r-tPA was approx. $(4-5) \times 10^{13} \text{ i.u./mol}$. This value was independent of the proportions of single- and two-chain enzyme present in the preparation, although the molar specific amidolytic activities of the two forms against S-2288 were significantly different. APNAN has also been used as a titrant for urokinase and human plasmin,

and operational molarities derived by using APNAN and NpGB were in good agreement (results not shown). APNAN is about 2.5-fold less sensitive than NpGB as a titrant at physiological pH. However, the inverse ester may prove useful for the estimation of purified t-PA solutions $> 5 \mu\text{M}$ because, unlike fluorogenic titrants, it can be used with relatively unsophisticated equipment and is simpler and less hazardous to use than radiolabelled titrants such as $[^3\text{H}]$ di-isopropyl phosphorofluoridate.

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