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REACTION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR WITH 4-METHYLUMBELLIFERYL-P-GUANIDINOBENZOATE HYDROCHLORIDE

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Summary: It has recently been reported that the fluorogenic serine proteolytic active site titrant, 4-methyl-umbelliferyl-p-guanidinobenzoate (MUGB), cannot be employed in this capacity for tissue-type plasminogen activator (TPA) [Geiger, M., and Binder, B.R. (1987) *Biochim. Biophys. Acta 912,* 34-40]. Since this observation has such important ramifications in this area of research, we have studied the reaction of MUGB with recombinant (rec)TPA under a variety of experimental conditions and find that MUGB is indeed an effective titrant of rec-two chain TPA (recTCTPA) at 4°, a condition under which the deacylation rate constant is diminished to the point that acylation can be readily observed. The K_S for the interaction of MUGB with recTCTPA is 43 μM - 46 μM, the acylation rate constant, k_2, is approximately 3.6 min^{-1} - 4.2 min^{-1}, and the rate constant for deacylation of p-guanidinobenzoyl-recTCTPA is 0.084 min^{-1} - 0.110 min^{-1}. This same recTCTPA, after treatment with diisopropylfluorophosphate, does not react with MUGB. Single-chain TPA (recSCTPA) has been found to acylate more slowly than its two-chain counterpart and to exhibit a higher degree of turnover of the acyl-enzyme with this reagent.

These results demonstrate that the active site concentration of TCTPA can be accurately determined by titration with MUGB, a consideration which is essential to the proper kinetic evaluation of this agent and its genetic variants. On the other hand, the presteady state kinetic characteristics for MUGB toward SCTPA are not favorable for its use as a titrant with this form of the enzyme.

Tissue plasminogen activator (TPA) is a serine protease that functions in the activation of human plasminogen to plasmin; this latter enzyme being responsible for lysis of the fibrin blood clot. TPA is synthesized as a single chain protein (SCTPA), containing 527 amino acids and four potential sites of glycosylation, only three of which are utilized (1). Upon exposure to other serine proteases, such as plasmin or trypsin, single-chain TPA is converted to its disulfide-linked, two-chain analogue (TCTPA), as a result of cleavage of the Arg^{275}-Ile^{276} peptide bond (1, 2). Whereas

Abbreviations used: TPA, tissue plasminogen activator; SCTPA, single chain tissue plasminogen activator; TCTPA, two chain tissue plasminogen activator; rec, recombinant; NPGB, p-nitrophenyl-p'-guanidinobenzoate; MUGB, 4-methyl-umbelliferyl-p-guanidino benzoate; $NaDodSO_4$, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

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TCTPA is more active toward small molecular weight substrates and more reactive with plasma inhibitors than SCTPA (3), these proteins have similar activities toward activation of human plasminogen (4).

Examination of the amino acid sequence of TPA has led to the observation that this protein consists of various domain regions. In addition to the signal peptide and the propeptide, similar to those of serum albumin (5), the molecule contains a 'finger' domain, homologous to those in fibronectin (6), a growth factor domain, resembling that of human epidermal growth factor (6), two 'kringle' regions homologous to those found in human plasminogen (1), and a region similar to trypsin and chymotrypsin, which contains the serine protease catalytic triad, at His^{322}, Asp^{371}, Ser^{478} (1, 7).

The plasminogen activating ability of TPA is greatly enhanced in the presence of fibrin (8), certain degradation products of fibrinogen (9), and, to a lesser extent, fibrinogen (10). These observations have led to an intensive study of TPA as a potential fibrin-specific clot lytic agent, with high therapeutic potential. Several commercial preparations of TPA exist, prepared *via* recombinant DNA routes, and a high number of genetic variant proteins have also been prepared. In order to fruitfully compare the kinetic properties of these proteins with each other, and with naturally occurring materials, it is imperative to be able to determine their active site concentrations. Effective serine protease titrants such as NPGB (11) and MUGB (12) exist, and are potentially useful for this purpose. The latter titrant is especially interesting since its hydrolysis leads to liberation of a fluorescent product, which allows titration of very low concentrations of TPA. This feature is useful for screening small amounts of genetic variants, prior to their mass production, and is necessary because of the poor solubility of TPA. However, it has recently been reported (13) that MUGB could not be employed as a titrant of TPA, due to its high level of turnover. Since the potential scientific value of this reagent is very high, we have decided to study more thoroughly the possibility of its use as a titrant for TPA. This communication presents the major conclusions of this work.

Materials and Methods

Proteins. The TPA preparations employed were prepared by recombinant DNA technology and were provided by Genentech, Inc. and the Monsanto Company. $NaDodSO_4$/PAGE analysis of the materials received, under reducing conditions, revealed that they were at least 80% in the single chain form. The recTPA variants used here, one containing only the 'kringle' 2 region, linked to the light chain (protease) region of TPA [des (6-175)]-recTPA, and another containing an Arg^{275}-Ser ([Ser^{275}]-recTPA) mutation, were also gifts of the Monsanto Company. These materials were present predominantly (>95%) as single polypeptide chains.

When desired, the Genentech and Monsanto rec-SCTPA preparations were converted to the respective recTCTPA as a consequence of treatment with Sepharose-plasmin, at a 10:1 (mole/mole) ratio of recSCTPA:plasmin. Pilot gel studies, employing $NaDodSO_4$/PAGE, under reducing conditions, were used to evaluate the most suitable reaction conditions, which were usually approximately 1 hr at room temperature.

Determination of presteady state kinetic constants for the recTPA-catalyzed hydrolysis of MUGB. An aliquot from a stock solution of 5 mM MUGB in dimethylformamide was diluted to 2 ml with a buffer composed of 50 mM Hepes-NaOH/100 mM NaCl, pH 7.4 and placed in a cuvette, such that a variety of concentrations, ranging from 5 μM to 50 μM were obtained. In each case, the spontaneous rate of hydrolysis of the substrate was monitored for approximately 2 min in a Perkin-Elmer MPF-44 fluorescence spectrometer. The cells were maintained at 4°. An excitation wavelength of 365 nm and an emission wavelength of 445 nm was used for analysis of the reaction. The slit width for both channels was 10 nm. Following this time, an aliquot of a stock solution of the relevant TPA, dissolved in a buffer of 10 mM Hepes/0.01 % (w/w) Tween-80, was added to the sample cuvette, and the same volume of buffer was added to the reference cuvette. The reaction was continually monitored until a linear rate of appearance of product occurred. The linear portion of the curve was extrapolated to zero time, and the displacement of the nonlinear portion from the extrapolated linear region of the experimental curve was employed as a measure of the 'burst' reaction.

The kinetic constants that characterize the interaction of MUGB and recTPA were calculated based on the acyl-intermediate scheme for serine proteases developed by Bender *et al.* (14). Our exact procedures have been detailed earlier (15). Briefly, the above displacement values were converted to concentrations of product liberated, by calibration of the recorder with a solutions of 4-methylumbelliferol. The apparent first order rate constant of acylation (b) was determined in the usual manner, and plotted against the initial concentration of MUGB in double reciprocal form. The dissociation constant (K_S) and first order rate constant for acylation that characterize the reaction of MUGB and recTPA, at 4°, was determined from the ordinate-intercept ($1/k_2$) and slope (k_S/K_2) of this latter plot, respectively.

Determination of the deacylation rate constant for p-guanidinobenzoyl-recTPA. The relevant recTPA was fully acylated by treatment of this enzyme with 100 μM MUGB, for 10 min, as described above. The acyl-enzyme was then diluted approximately 100-fold into an assay cuvette, maintained at 4°, containing the TPA steady state substrate, H-D-Ile-Pro-Arg-p-nitroanilide (S-2288). The absorbance at 405 nm, resulting hydrolysis of this substrate, was measured continuously, and is proportional to the amount of deacylated recTPA present. The reaction was allowed to proceed until a limiting reaction rate was reached. The k_3 value was calculated as the reciprocal of the intercept on the abscissa, of the linear portion of the graph.

Results

Figure 1 illustrates the reaction of recTCTPA, recSCTPA and diisopropylfluorophosphate-inactivated recTCTPA (DIP-recTCTPA) with MUGB at 4°. Clearly, in the case of recTCTPA, a rapid reaction, corresponding to liberation of approximately 0.7 moles of methyl umbelliferol per mole of recTPA, is seen. Subsequent to this rapid release of product, a much slower rate of hydrolysis of MUGB occurs. Comparatively, DIP-recTCTPA does not catalyze significant hydrolysis of MUGB, showing that the active site in recTCTPA is needed for the reaction. The results suggest that $k_2 >> k_3$ for the reaction of MUGB with recTCTPA and that the MUGB is essentially a single turnover substrate, which can be fruitfully employed as a titrant for active recTCTPA. RecSCTPA reacts with MUGB more slowly than recTCTPA.

The presteady state kinetic parameters, at 4°, for the reaction of MUGB with recTCTPA, obtained as the single-chain form from two different sources, were

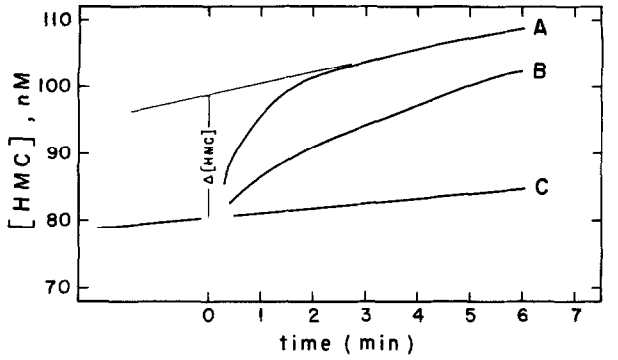

Figure 1. Reaction of MUGB with recTPA at 4°. **A.** A quantity of 20 μM MUGB was added to a cuvette containing 34 nM recTCTPA (Genentech material). **B.** As in A, except that 34 nM recSCTPA was employed as the protein. **C.** As in A, except that the recTCTPA was first treated with diisopropylfluorophosphate, until <5% of its amidase activity toward the substrate, S-2288, was obtained. The buffer was 50 mM Hepes-NaOH/100 mM NaCl, pH 7.4. The ordinate was calibrated by measuring the fluorescence of known levels of 4-methylumbelliferol [hydroxymethyl coumarin (HMC)].

determined in order to demonstrate quantitatively that MUGB is a virtual single turnover substrate. A typical example of the plots of the apparent first order rate constant for acylation of recTCTPA by MUGB against the concentration of MUGB is displayed in Figure 2 for the Genentech material. From the ordinate-intercepts and slopes, respectively, the k_2 and K_S values were determined. The Genentech material yielded a k_2 of 3.6 ± 0.4 min^{-1} and a K_S of 46 ± 8 μM and these same constants for the Monsanto recTCTPA were 4.2 ± 0.4 min^{-1} and 43 ± 7 μM, respectively. We have also evaluated the presteady state kinetic constants for a genetic variant of recTPA, [des (6-175)]-recTCTPA, which contains only the 'kringle' 2 domain, linked to the serine protease region. The presteady state kinetic parameters found for the MUGB reaction with [des (6-175)]-recTCTPA were 5.4 ± 0.5 min^{-1} and 67 ± 9 μM for k_2 and K_S, respectively.

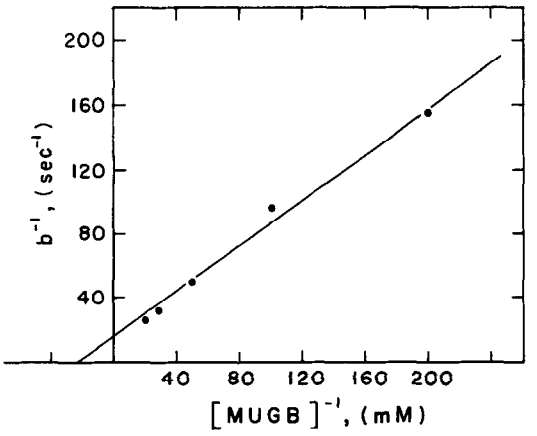

Figure 2. A plot of the apparent first order rate constant for acylation (b) of recTCTPA (Genentech material) against the concentration of MUGB at 4°.

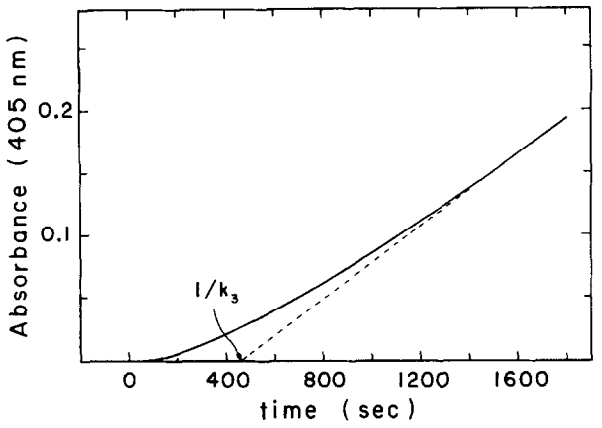

Figure 3. The rate regeneration of activity of guanidinobenzoyl-recTCTPA (Genentech material) at 4°. The activity of guanidinobenzoyl-recTCTPA was measured by incubation of the protein with the steady state chromogenic substrate, S-2288 (0.5 mM), and the absorbance at 405 nm, due to release of p-nitroanilide from the substrate, was continuously monitored. The deacylation rate constant is equal to the reciprocal of the abscissa-intercept of the linear portion of the curve.

We have also attempted to determine the presteady state kinetic constants for recSCTPA, we found that acylation and deacylation rates were not sufficiently different for the presteady state rate theory to apply. In order to evaluate this point further, we performed the same experiments on the genetic variant, [Ser^{275}]-recSCTPA, containing a substitution at the cleavage site. This material behaved as the normal recSCTPA, wherein acylation and deacylation rates with MUGB did not show proper characteristics for employment of this agent as a titrant.

The deacylation rate constants, at 4°, for each of the above recTPA preparations were determined by assay of the rate of recovery of their respective amidase activities toward the steady state substrate, S-2288. An example of the data obtained is shown in Figure 3 for the Genentech recTCTPA. Analysis of the data reveals a deacylation rate constant for p-guanidinobenzoyl-recTCTPA of 0.084 ± 0.01 min^{-1}. Similarly, the Monsanto recTCTPA yielded a k_3 value of 0.11 ± 0.01 min^{-1}, while that for [des (6-175)]-recTCTPA was 0.126 ± 0.02 min^{-1}.

A summary of all presteady state kinetic constants that characterize the interaction of MUGB with recTPA, at 4°, is provided in Table I.

Table I

Protein	k_2 (min^{-1})	K_S (μM)	k_3 (min^{-1})	k_2/K_S ($min^{-1}\mu M^{-1}$)
recTCTPA (Genentech)	3.6 ± 0.4	46 ± 8	0.084 ± 0.010	0.078
recTCTPA (Monsanto)	4.2 ± 0.4	43 ± 7	0.110 ± 0.010	0.098
[des (6-175)]-recTCTPA	5.4 ± 0.5	67 ± 9	0.126 ± 0.020	0.081

Discussion

Previous work has demonstrated that MUGB is an effective active site titrant for serine proteases such as α-chymotrypsin (12), trypsin (12), thrombin (12), factor Xa (12), and urokinase (16), and we routinely employ this reagent for titration of plasmin. While titrants, such as NPGB and 2-nitrophenyl-4'-anisate (17), exist for TPA, a sensitive fluorogenic reagent for evaluation of operational molarities of TPA is highly desirable for ease in comparison of kinetic properties of different recombinant or cell-derived TPA preparations, when present at very low concentrations. Although MUGB is a prime candidate for such a titrant, it has recently been claimed that this agent cannot be employed for this purpose since it was believed not to generate a stable acyl-enzyme complex with neither SCTPA nor TCTPA (13). Due to the importance of this issue, we have carefully evaluated the kinetic constants of MUGB with a variety of TPA preparations, and found that conditions exist wherein MUGB can be very effectively utilized as a titrant for TPA and various TPA derivatives.

Interaction of recTCTPA with MUGB, at 4°, reveals a rapid liberation of methyl umbelliferol (HMC), followed by slow turnover of this substrate, typical of a reaction wherein acylation rates are much faster than deacylation rates. The active site serine residue of recTCTPA is necessary for this phase of the reaction. In order to demonstrate whether MUGB can be effectively employed as an active site serine titrant for this protein, we have evaluated the kinetic constants for its interaction with recTCTPA, which are listed in Table I. With two different sources of recTCTPA, the k_2/k_3 is approximately 38 (Monsanto) and 43 (Genentech). This shows that MUGB possesses the properties of a suitable titrant for this enzyme, at least at 4°. The data of Figure 1 illustrate that quantities of recTCTPA as low as 20 nM could be readily titrated with MUGB, and that titration of smaller amounts are certainly possible.

Since much hope rests on TPA as a fibrin-specific thrombolytic agent, accurate determination of the active concentration of TPA is needed for meaningful comparisons of the kinetic properties of the many recombinant preparations that are now available, and for evaluation of genetic variants of this protein as possible second generation drugs. In order to screen the kinetic properties of variant proteins prior to large-scale production, and because of the poor solubility of TPA under physiological conditions, a titrant capable of operation at very low concentrations of this protein is needed. MUGB is at least 100-fold more sensitive than agents such as NPGB for this purpose, and could serve extremely well in this capacity. For the recTCTPA-variant that we have examined, *i.e.,* [des (6-175)-recTCTPA], MUGB is an effective titrant (k_2/k_3 = 43). The similarity of the presteady state kinetic constants for this variant with normal recTCTPA suggest that the acylation and deacylation reactions are not dependent on the 'finger', growth factor and 'kringle' 1 regions of the molecule.

SCTPA possesses hydrolytic activity toward synthetic substrates (3, 18, 19), albeit to a lesser degree than TCTPA. Because of similar rates of acylation and

deacylation of the single chain form of TPA, we were not able to accurately evaluate its individual presteady state rate constants with MUGB. However, it is clear that greatly different enzymatic properties toward this small substrate are present in the single chain and two chain forms of TPA. As is clear from Figure 1, these differences are mainly revealed through a slower acylation rate and faster deacylation rate with SCTPA, as compared to TCTPA.

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