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A Comparison of Steady- and Presteady-State Kinetics of Bovine and Human Plasmins†

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ABSTRACT: A comparison of steady-state and presteady-state kinetics on plasmins derived from a streptokinase-insensitive plasminogen (bovine) and a streptokinase-sensitive plasminogen (human) has been undertaken. Steady-state kinetics on bovine plasmin using α-*N*-tosyl-L-arginine methyl ester as the substrate at 22° yielded K_m and V_{max} values of 8.2×10^{-3} M and 17.5 μmoles min^{-1} mg^{-1}, respectively. Benzamidine hydrochloride was found to be a competitive inhibitor of plasmin esterase activity with a K_I value of 5.1×10^{-4} M. The corresponding values for human plasmin were 7.7×10^{-3} M, 12.4 μmoles min^{-1} mg^{-1}, and 10.6×10^{-4} M. Presteady-state kinetics using *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride as the substrate for bovine plasmin at 22° yielded k_2 and K_s values of 0.543 sec^{-1} and 22.2×10^{-6} M and for human plasmin the corresponding values were 0.064 sec^{-1} and 5.42×10^{-6} M. Similar presteady-state experiments were performed with another acylating agent, *N*-(*p*-carboxybenzyl)pyridinium *p*-nitrophenyl ester bromide. This reagent yielded k_2 and K_s values for bovine plasmin at 22° of 0.085 sec^{-1} and 2.12×10^{-3} M and values of 0.084 sec^{-1} and 5.34×10^{-4} M for human plasmin. These studies indicate that marked differences occur in the active sites of bovine and human plasmins.

Plasmin (EC 3.4.4.14) is a proteolytic enzyme which functions, physiologically, in the dissolution of the fibrin clot and exists in the plasma as the inactive precursor, plasminogen. The conversion of plasminogen to plasmin has been widely studied and has some well recognized features. Activation of human plasminogen by a urinary protease, urokinase, or by a variety of other tissue proteases proceeds through cleavage of a single arginyl–valine bond in plasminogen, yielding a two-chain plasmin structure stabilized by a single disulfide bond (Robbins *et al.*, 1965, 1967; Summaria *et al.*, 1967a,b; Groskopf *et al.*, 1969a). Activation of human plasminogen also occurs with a bacterial endotoxin, streptokinase. However, since streptokinase possesses no inherent proteolytic activity its induced cleavage of a peptide bond in plasminogen occurs through its combination with "proactivator" and subsequent formation of "activator," which possesses the necessary proteolytic activity for cleavage of a peptide bond in plasminogen (see Kline and Fishman (1963), Davis *et al.* (1964), Ling *et al.* (1967), Summaria *et al.* (1969), and Reddy and Marcus (1972)). It has also been found (see Wulf and Mertz, 1969) and in part confirmed on purified plasminogens (Brockway and Castellino, 1972) that streptokinase is species specific, only activating the plasminogens of man, monkey, and cat, whereas urokinase or "activator" activated the plasminogens of all species so far studied.

The plasmin obtained by activating plasminogen as described above is a proteolytic enzyme which possesses trypsin-like esterase activity, catalyzing the hydrolysis of α-NH_2-substituted lysine and arginine esters (Sherry *et al.*, 1966). Human plasmin possesses an active-site serine and histidine

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residue (Summaria *et al.*, 1967a; Groskopf *et al.*, 1969a,b), suggesting that plasmin is a typical serine protease. Plasmin also undergoes the NphBzoGdn[1] burst reaction, indicating that the enzyme proceeds through an acyl-enzyme intermediate in its catalytic activity. This mechanism is common to trypsin, thrombin, chymotrypsin, etc.

We are in the process of studying the esterolytic properties of various plasmins in order to compare, kinetically, plasmins derived from streptokinase-sensitive and streptokinase-insensitive species and also to compare the kinetics of various plasmins to other proteolytic enzymes such as trypsin and thrombin. This manuscript presents our studies on kinetic comparisons between bovine and human plasmins.

Materials and Methods

Proteins. Human plasminogen was prepared from Cohn fraction III, generously donated by Cutter Laboratories, by affinity chromatography as previously described (Brockway and Castellino, 1972), and bovine plasminogen was prepared from whole citrated plasma obtained fresh from a local packing plant, in the same manner. In addition each plasminogen (4 ml) was passed through a 2.5 × 60 cm column of Sephadex G-100 and only one major peak was eluted for bovine plasmin and a small impurity was separated from human plasminogen. Urokinase was purchased from Calbiochem and possessed a specific activity of 40,000 CTA units mg^{-1}. Streptokinase (varidase) was purchased from a local drug outlet and purified as described by DeRenzo *et al.* (1967). The final specific activity was approximately 100,000 units mg^{-1}.

Substrates and Inhibitors. Tos-Arg-OMe was purchased from Cyclo Chemical Co., NphBzoGdn was synthesized as described by Chase and Shaw (1970), and NphTolPdn was generously donated to us by Dr. Elliot Shaw. Benzamidine·HCl was purchased from Aldrich Chemical Co.

Other reagents were the best commercially available and used without further purification.

Activation of Plasminogen. Human plasmin was obtained by incubating a concentrated solution (~15 mg/ml) of plasminogen in 0.05 M Tris·HCl–15% glycerol (pH 8.2) with 100 units/ml of urokinase or 100 units/ml of streptokinase at room temperature. The formation of plasmin was monitored by the burst assay using NphBzoGdn (Chase and Shaw, 1969) and was found to be complete in approximately 2 hr. The amount of active plasmin at this stage corresponded to approximately 80% of the added plasminogen in each case as determined by the *p*-nitrophenol burst assay. Analysis of the plasmins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated complete conversion of the plasminogens to plasmins. At this point each plasmin was placed in an ice bath to inhibit autocatalytic destruction. The same procedure for activation of bovine plasminogen was used with urokinase as the activator except that slightly higher amounts of urokinase were employed (~700 units/ml).

Steady-State Kinetic Parameters. K_m and V_{max} values for Tos-Arg-OMe on human and bovine plasmin were obtained as described earlier (Brockway and Castellino, 1971) except that all values are expressed in terms of active plasmin, the amount of which was determined in the stock solution by the burst assay. The amount of active plasmin in each assay tube was decreased to 10–15 μg. The K_I values of benzamidine·HCl were determined by competition with Tos-Arg-OMe in the usual manner.

Determination of k_2 and K_s. Kinetic expressions for the titration of enzymes which proceed through an acyl-enzyme intermediate have been developed (Bender *et al.*, 1966; Bender and Brubacker, 1966).

The catalytic sequence is

$$E + S \overset{K_s}{\rightleftarrows} E\cdot S \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P' \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad +$$
$$\qquad\qquad\qquad\qquad\quad P$$

where E is the enzyme, S is the ester substrate, E·S is the adsorptive complex, P is the first released product (alcohol moiety), ES′ is the acyl-enzyme intermediate, and P′ is the second released product (acid moiety). In this scheme k_2 is the rate constant for acylation of the enzyme and k_3 is the deacylation rate constant.

When $[S_0] > [E_0]$, the integrated rate of formation of the acyl-enzyme is (Bender *et al.*, 1966)

$$-bt = \ln \frac{[E_0] - [1 + (k_3/k_2)(1 + K_s/[S_0])][ES']}{[E_0]} \qquad (2)$$

For the reaction of NphBzoGdn and NphTolPdn with plasmin, $k_2 >>> k_3$ and eq 2 can then be reduced to

$$\frac{1}{b} = \frac{1}{k_2} + \frac{K_s}{k_2[S_0]} \qquad (3)$$

where b is an apparent first-order rate constant for formation of acyl-enzyme and $[S_0]$ is the initial substrate concentration.

The presteady-state reaction for NphBzoGdn and plasmin occurs much too rapidly to be observable by ordinary techniques. Consequently, we have slowed the presteady state by addition of a competitive inhibitor of plasmin, benzamidine·HCl. In the presence of the competitive inhibitor eq 3 becomes

$$\frac{1}{b} = \frac{1}{k_2} + \frac{K_s(1 + [I]/K_i)}{k_2[S_0]} \qquad (4)$$

where [I] is the concentration of benzamidine·HCl used and K_I its corresponding binding constant. Thus, k_2 and K_s can be obtained from a plot of $1/b$ *vs.* $1/[S_0]$ at constant [I] with prior knowledge of K_I.

The effectiveness of benzamidine·HCl in slowing the presteady state of human and bovine plasmin with NphBzoGdn can be derived from Figure 1 by observing the time scale over which the reaction occurred. Also from Figure 1, it is evident that with each plasmin the reaction is first order over the entire range of NphBzoGdn concentrations. These studies were carried out at the desired temperature by adding 0.100–0.700 ml of NphBzoGdn in 2% dimethylformamide–0.05 M Veronal (pH 8.3) (working range of substrate 1×10^{-5}–1×10^{-4} M) and 0.100 ml of 0.10 M benzamidine·HCl in 0.05 M Veronal (pH 8.3). Veronal buffer was used to adjust the volume at this point to 0.800 ml. The reaction was initiated by addition of 0.04 ml of plasmin, activated as described above, and monitored at 410 mμ in a Cary Model 15 spectrophotometer with a circulating-temperature control. Values

[1] Abbreviations used are: NphBzoGdn, *p*-nitrophenyl *p*′-guanidinobenzoate; NphTolPdn, *N*-(*p*-carboxybenzyl)pyrinidinium *p*-nitrophenyl ester. The trivial name for this compound, nitrophenyltoluoylpyridine, was used in assigning the abbreviation. Tos-Arg-OMe, α-*N*-tosyl-L-arginine methyl ester.

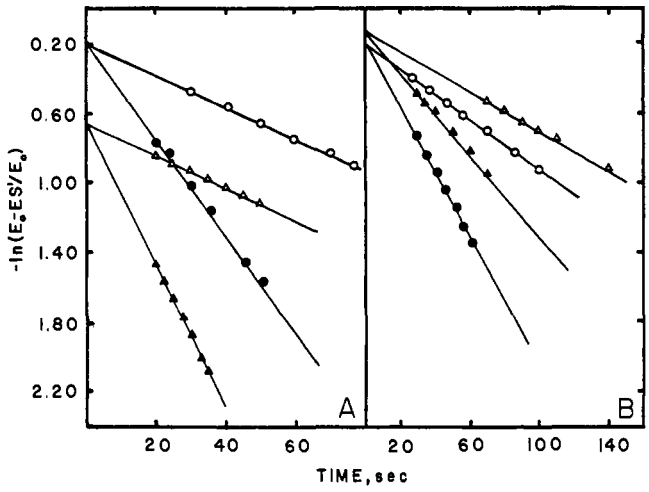

FIGURE 1: First-order kinetic plots for the presteady-state reaction of human and bovine plasmins with NphBzoGdn and NphTolPdn. (A) Plots of the natural logarithm of the relative amount of remaining plasmin as a function of time of reaction with NphBzoGdn. The conditions illustrated are: (○) 2.52×10^{-6} M human plasmin, 9.95×10^{-6} M NphBzoGdn. 1.11×10^{-2} M benzamidine hydrochloride; (●) 2.52×10^{-6} M human plasmin, 4.98×10^{-5} M NphBzoGdn, 1.11×10^{-2} M benzamidine hydrochloride; (△) 2.72×10^{-6} M bovine plasmin, 9.26×10^{-6} M NphBzoGdn, 1.16×10^{-2} M benzamidine hydrochloride; (▲) 2.72×10^{-6} M bovine plasmin, 4.63×10^{-5} M NphBzoGdn, 1.16×10^{-2} M benzamidine hydrochloride. For each plasmin the NphBzoGdn concentrations listed represent the lowest and highest concentrations used in our presteady-state calculations. (B) Plots of the natural logarithm of the relative amount of remaining plasmin as a function of time of reaction with NphTolPdn: (○) 4.40×10^{-6} M human plasmin, 4.24×10^{-5} M NphTolPdn; (●) 4.40×10^{-6} M human plasmin, 1.70×10^{-4} M NphTolPdn; (△) 3.55×10^{-6} M bovine plasmin, 1.62×10^{-4} M NphTolPdn; (▲) 3.55×10^{-6} M bovine plasmin, 3.48×10^{-4} M NphTolPdn. For each plasmin the NphTolPdn concentrations listed represent the lowest and highest concentrations used in our presteady-state calculations.

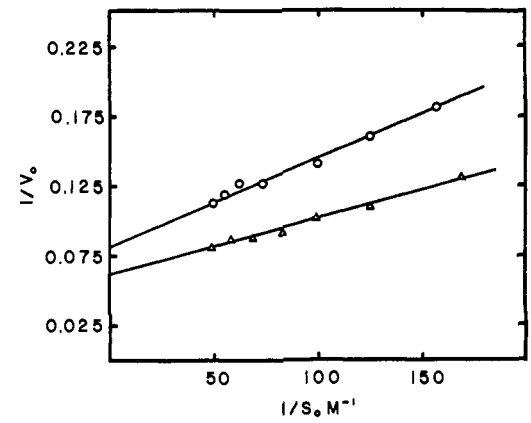

FIGURE 2: Steady-state analysis of human and bovine plasmins at 22° with Tos-Arg-OMe. V_0 is expressed as μmoles of Tos-Arg-OMe cleaved min^{-1} mg^{-1} of active plasmin. (○) Human plasmin and (△) bovine plasmin.

of b for a series of NphBzoGdn concentrations were obtained from a series of plots performed as in Figure 1.

The values of k_2 and K_s for NphTolPdn were determined in a similar manner by use of eq 3 directly, since as shown by Glover *et al.* (1971) the presteady state with this compound is so slow that addition of an inhibitor is not necessary. Figure 1 also provides examples to illustrate this point with human and bovine plasmin. Values of b can be obtained from the slopes of plots as in Figure 1 and are first order over the entire concentration range. Values of k_2 can be obtained from the intercept of a plot of $1/b$ *vs.* $1/[S_0]$ and K_s can be obtained from the slope of the same plot.

Results

Steady-state kinetic experiments were performed on bovine and human plasmins with Tos-Arg-OMe as the substrate. The results are plotted in double-reciprocal form in Figure 2. These plots were used to obtain K_m and V_{max} values for Tos-Arg-OMe on each plasmin. The binding constant, K_I, for benzamidine·HCl on each plasmin was obtained by competition with Tos-Arg-OMe. The results of these experiments for bovine and human plasmin are presented in Figure 3. Values of K_m, V_{max}, and K_I are summarized in Table I. Clearly, benzamidine is a competitive inhibitor of Tos-Arg-OMe hydrolysis by each plasmin.

Plots of $1/b$ *vs.* $1/[S_0]$ for NphBzoGdn and each plasmin in the presence of benzamidine·HCl are presented in Figure 4. The values of k_2 and K_s were obtained from the $-y$ intercepts and slopes of each plot by use of eq 4 and the results are listed in Table I. The values of k_3 were so low that they could not be accurately measured. However, since k_3 is essentially a measure of V_{max} we feel that the values of V_{max} for Tos-Arg-OMe, a substrate which exhibits a much more rapid rate of deacylation, would suffice for a comparison of k_3 values in the two plasmins.

Plots of $1/b$ *vs.* $1/[S_0]$ for NphTolPdn and each plasmin in the absence of any inhibitor of acylation are also presented in Figure 4. The values of k_2 and K_s were obtained from the

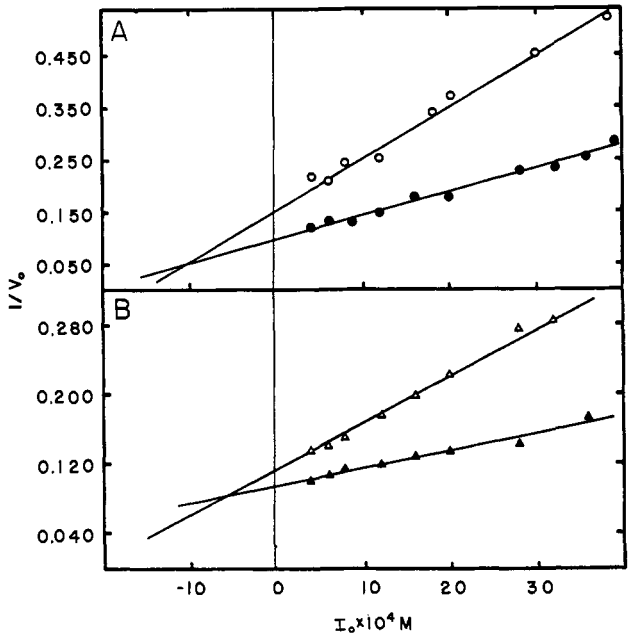

FIGURE 3: Determination of the K_I for benzamidine·HCl on plasmin at 22°. V_0 represents the initial velocity, expressed as in Figure 2, of a given amount of plasmin at a constant concentration of L-Tos-Arg-OMe in the presence of various concentrations of benzamidine hydrochloride (I). The point of intersection of the two lines is equal to $-K_I$ for benzamidine. (A) (○) 8.0×10^{-3} M Tos-Arg-OMe, (●) 1.6×10^{-2} M Tos-Arg-OMe; human plasmin. (B) (△) 8.0×10^{-3} M Tos-Arg-OMe, (▲) 1.6×10^{-2} M Tos-Arg-OMe; bovine plasmin.

TABLE I: Steady- and Presteady-State Parameters for Human and Bovine Plasmins at 22°.

Parameter	Bovine	Human
K_m[a] (M)	$8.2 \pm 0.4 \times 10^{-3}$	$7.7 \pm 0.4 \times 10^{-3}$
V_{max}[a,b]	17.5 ± 0.5	12.4 ± 0.5
K_i[a] (M)	$5.1 \pm 1.0 \times 10^{-4}$	$10.6 \pm 1.0 \times 10^{-4}$
k_2[c] (sec^{-1})	0.543 ± 0.070	0.064 ± 0.010
K_s[c] (M)	$22.2 \pm 2.0 \times 10^{-6}$	$5.4 \pm 1.0 \times 10^{-6}$
k_2/K_s[c] ($sec^{-1}\ M^{-1}$)	24,460	11,850
k_2[d] (sec^{-1})	0.085 ± 0.010	0.084 ± 0.010
K_s[d] (M)	$2.12 \pm 0.40 \times 10^{-3}$	$5.34 \pm 0.60 \times 10^{-4}$
k_2/K_s[d] ($sec^{-1}\ M^{-1}$)	40.1	150.7

[a] Values for Tos-Arg-OMe. [b] Units of μmoles of Tos-Arg-OMe cleaved min^{-1} mg^{-1} of active plasmin. [c] Values for *p*-nitrophenyl *p'*-guanidinobenzoate. [d] Values for *N*-(*p*-carboxybenzyl)pyridinium *p*-nitrophenyl ester.

$-y$ intercepts and slopes of each plot by use of eq 3. The results are also listed in Table I.

Discussion

The steady-state and presteady-state kinetic data obtained in this study indicate that some major differences exist in the active sites of bovine and human plasmins. In the less discriminating steady-state parameters the K_m values are approximately equal for the two enzymes. However, since the K_m is a complex constant, reflecting values of several rate constants, a great significance should not be attached to the equality of this parameter. On the other hand, it is evident from Table I that the V_{max} values for Tos-Arg-OMe are considerably different for the two plasmins. Since the V_{max} in these cases is a reflection of k_3, more significance could be attached to differences in this parameter. A possible interpretation of these differences is that in the acyl-enzyme intermediate, tosylarginyl-plasmin, the tosylarginine moiety exists in different geometries on each plasmin, allowing more susceptibility to deacylation of the complex with bovine plasmin than with human plasmin. Further differences in the two enzymes can be noted in Table I in that the binding of a competitive inhibitor, benzamidine·HCl, to bovine plasmin is approximately twice as strong as its binding to human plasmin. Our values for the K_m and V_{max} of Tos-Arg-OMe and human plasmin are similar to those reported by Robbins *et al.* (1965) and to those reported by us earlier (Brockway and Castellino, 1971).

Significant, and more interpretable, differences in the two enzymes are evident in consideration of the presteady-state parameters. With NphBzoGdn, the acylation rate constant, k_2, is approximately 8.5-fold higher for bovine plasmin than for human plasmin whereas K_s is 4-fold lower for human plasmin than for bovine plasmin. These results indicate that the orientation of NphBzoGdn on the enzyme surface of bovine plasmin is in a more favorable position for nucleophilic attack by the active site serine than for human plasmin.

Values of k_2/K_s for NphBzoGdn on each plasmin can be obtained from the data presented in this study and are listed in Table I. As pointed out initially by Peller and Alberty (1959) the maximum value for this parameter is equal to 10^8–10^{10} M^{-1} sec^{-1} and is attained when the bimolecular interaction between substrate and enzyme is controlled only by their rates of diffusion. Since our values of k_2/K_s are significantly lower than the diffusional upper limit, it is doubtless that geometric factors of fitting enzyme and substrate together at least partially account for this behavior. This rationale has been previously applied by Bender and Kezdy (1965). With this in mind it can be seen from Table I that NphBzoGdn is better geometrically oriented to the active site of bovine plasmin than to human plasmin and the reasons for this lie in both k_2 and K_s considerations, indicating the differences in the active site of these plasmins. Unfortunately, there are not many plasmin substrates for which values of k_2/K_s have been determined. Therefore, additional comments on the active-site specificity of plasmin must be postponed until further studies are reported for other compounds. The k_2/K_s value of 24,460 for NphBzoGdn and bovine plasmin obtained in this study is very different from the value of 3.2×10^7 for NphBzoGdn and bovine trypsin but is similar to the value of 32,910 for NphBzoGdn and bovine thrombin (Chase and Shaw, 1969). The value of k_2/K_s of 11,850 for human plasmin and NphBzoGdn obtained in this study compares favorably to the value of 18,820 for human thrombin and NphBzoGdn (Chase and Shaw, 1969). These results suggest that the active site for NphBzoGdn on plasmin is more similar to that site on thrombin than for trypsin, although significant differences exist in all comparisons.

It should be pointed out that the values for k_2 and K_s for NphBzoGdn and human plasmin differ significantly from the values reported for the same system by Chase and Shaw (1969). These authors performed their study in the absence of any inhibitor of acylation and have reported that the final stages of the presteady state are directly observable under

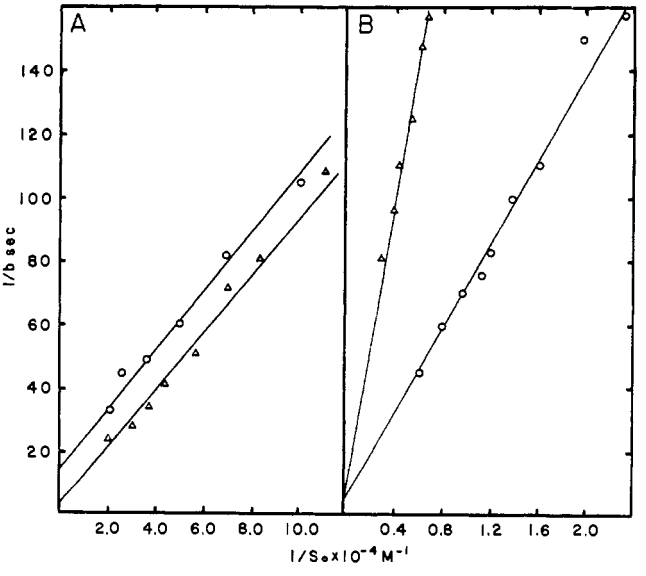

FIGURE 4: Determination of k_2 and K_s values for bovine and human plasmins at 22°. Apparent first-order rate constants (*b*) are plotted for a series of concentrations of substrate [S_0]. (A) Plasmins and NphBzoGdn. Equation 4 was used for determination of k_2 and K_s. The K_I value of benzamidine hydrochloride was determined from Figure 3. (○) 2.52×10^{-6} M human plasmin, 1.11×10^{-2} M benzamidine hydrochloride; (△) 2.72×10^{-6} M bovine plasmin, 1.16×10^{-2} M benzamidine hydrochloride. (B) Plasmins and NphTolPdn. Equation 3 was used for determination of k_2 and K_s. (○) 4.40×10^{-6} M human plasmin; (△) 3.55×10^{-6} M bovine plasmin.

the conditions where $[S_0]$ is only slightly greater than $[E_0]$. They have performed their experiments on this basis and have obtained a value of k_2 of 0.46 sec^{-1} and a K_s value of 11.3×10^{-6} M. Under these conditions, we observed only the final portion of the presteady state which was not adequate for calculations and the validity of the first-order assumption becomes questionable. Furthermore, the lower NphBzoGdn concentrations used by these authors indicate a maximum burst of 0.02–0.03 assuming complete acylation of the plasmin. Our burst sizes are approximately two times their values in a given experiment. Consideration of the small burst sizes and the fact that the half-lives of their measured rates were in the range of 3–10 sec leads us to believe that a large error is inherent in their measurements. We feel that the k_2 and K_s obtained in this study are the more correct values for NphBzoGdn and human plasmin.

An entirely different picture is noted when comparing the presteady-state parameters of NphTolPdn to bovine and human plasmins. This reagent was synthesized by Glover *et al.* (1971) and found to acylate plasmin and thrombin at rates slower than NphBzoGdn. In this case, NphTolPdn is better oriented to the active site on human plasmin than on bovine plasmin; however, the acylation rate constants are similar for both plasmins. The main difference lies in the fact that the K_s for bovine plasmin is 4-fold higher than for human plasmin. The indication from these data is that subsequent to the binding process the substrate on each plasmin is equally susceptible to attack by the active center serine residue allowing displacement of the alcohol moiety of the ester substrate to occur at equal rates in the two plasmins. The two species of plasmin therefore respond differently to changes in the structure of the substrate and suggest that the steric requirements of each plasmin active site are inherently different. However, no detailed comparison can be made on the basis of only two substrates. Further work on this point to establish more details concerning these differences is currently in progress.

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