Scott, T. G., Spencer, R. D., Leonard, N. J., & Weber, G. (1970) *J. Am. Chem. Soc. 92*, 687–695.
Smith, E. L., Landon, M., Piskiewicz, D., Brattin, W. J., Langley, J. J., & Melamed, M. D. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 724–730.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.
Sund, H., Pilz, I., & Herbst, M. (1969) *Eur. J. Biochem. 7*, 517–525.
Wallis, R. B., & Holbrook, J. J. (1973) *Biochem. J. 133*, 173–182.

Interference of Active Site Specific Reagents in Plasminogen–Streptokinase Active Site Formation†

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ABSTRACT: We have recently observed slow, non-Michaelis–Menten kinetics of activation of native cat plasminogen by catalytic concentrations of streptokinase. In order to understand the reasons for this phenomenon, we undertook to study the formation of the plasminogen–streptokinase activator complex under the same plasminogen activation conditions. The results obtained in this study show that the potential active site in both cat and human plasminogen is capable of binding strongly the specific substrates (S) *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) and H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide, though the active site is incapable of hydrolyzing these substrates. Binding studies support these and the following conclusions. Streptokinase binds to this zymogen–substrate complex to create the ternary plasminogen–S–streptokinase complex, which then slowly converts to an acylated plasminogen–streptokinase form. This acylation reaction is 550 times slower than acylation of the preformed plasminogen–streptokinase complex by NPGB. The same reaction also occurs with human plasminogen, though the acylation reaction is 10 times faster than when the cat zymogen is used. NPGB binds specifically to plasminogen but not to streptokinase. These studies proved that inhibition of cat plasminogen activation by streptokinase occurs at the level of activator complex formation. We conclude from our studies that streptokinase binding to both cat and human plasminogen occurs at the potential active site of the zymogen. Consequently, it is probable that plasminogen activation in vivo is inhibited by binding of active site specific inhibitors to plasminogen.

The plasma zymogen plasminogen is activated to the corresponding serine protease enzyme form, plasmin, by proteolytic cleavage of its Arg_{560}–Val peptide bond. This reaction is catalyzed efficiently by urokinase or by a noncovalent binary complex formed by plasminogen with the bacterial protein streptokinase. Streptokinase itself has never been shown to possess any enzymatic activity. The equimolar plasminogen–streptokinase complex has a serine protease active site with a high specificity for activating plasminogen to plasmin via cleavage of the Arg_{560}–Val scissile bond. This active site, formed upon noncovalent complexing of the inactive zymogen with streptokinase, is in all respects a fully functional active site, and it can be titrated with *p*-nitrophenyl *p*-guanidinobenzoate (NPGB)[1] (McClintock & Bell, 1971; Reddy & Markus, 1972; Schick & Castellino, 1974). The active site generated in the complex resides in the plasminogen moiety (Morris et al., 1981), since the complex, once formed, can be dissociated and the plasminogen moiety is still active after separation from streptokinase (Summaria et al., 1982).

Both trypsinogen and chymotrypsinogen are able to bind and to hydrolyze ester substrates, including NPGB, by a mechanism identical with that of the corresponding enzyme. These reactions proceed orders of magnitude more slowly with the zymogen than with the enzyme (Gertler et al., 1974; Kerr et al., 1975; Robinson et al., 1975). Bode & Huber (1976) and Bode et al. (1978) showed that upon strong ligand binding by trypsinogen, there is a transition of the tertiary structure of the enzyme to a trypsin-like state. This transition could be induced in trypsinogen by specific short peptides which resemble the amino terminus of the enzyme or by strong active site inhibitors like pancreatic trypsin inhibitor.

We have previously reported on some properties of a plasminogen zymogen species with an active site which was isolated from Cohn fraction $III_{2,3}$ (Wohl et al., 1977). More recently, we reported on a plasminogen zymogen species with an active site, which was isolated after its active site was formed by complex formation with streptokinase (Summaria et al., 1982). These last reports verified that it is possible to have a functional active and binding site in the plasminogen zymogen molecule without hydrolysis of the Arg_{560}–Val peptide bond but gave no indication that such a binding site may be of importance in the regulation of plasminogen activation.

Previous studies also showed that the plasminogen–streptokinase complex is a noncovalent stoichiometric complex which is formed without any proteolytic cleavage (Robbins & Summaria, 1970). They did not show, however, how the active site is generated, or where the binding of streptokinase occurs. There are no detailed studies of the process of formation of the active plasminogen–streptokinase complex.

Our observation that cat plasminogen–streptokinase complex titration with NPGB is not instantaneous when streptokinase is added last to the reaction mixture provided us with a way

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[1] Abbreviations: IU, international unit(s), established by the World Health Organization for streptokinase; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; H-D-Val-Leu-Lys-pNA, H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide; Plg, plasminogen; SK, streptokinase.

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to study these processes. Since the complex formation is not instantaneous, we could examine each step in the enzymatic reaction sequence more closely. The first step in the reaction sequence is formation of the plasminogen–streptokinase activator complex. The approach taken in this study was to examine the process of activator complex formation via NPGB titration experiments.

Materials and Methods

Materials

Native human and cat plasminogens were isolated from pooled human and cat plasma, respectively, by previously described methods (Wohl et al., 1980, 1983). Both plasminogens had better than 95% active sites upon activation. Cat plasma was obtained from Pel-Freez Biologicals, Inc., Rogers, AR. The highly pure streptokinase preparation (10^5 IU/mg of protein) was a gift from Dr. R. Lundén, AB Kabi, Sweden.

All chemicals were reagent grade. H-D-Valyl-L-leucyl-L-lysyl-*p*-nitroanilide[1] (Friberger et al., 1978) was purchased from Helena Laboratories.

Methods

Pre-Steady-State Kinetic Studies. Experiments were performed in a Durrum-Gibson stopped-flow spectrophotometer with a 75-μL, 2-cm path-length cell. The cell was thermostated at 37 °C and monitored at 410 nm. Experiments were performed by mixing 40 μL of an equimolar preformed plasminogen–streptokinase solution (1×10^{-5} M) and 40 μL of NPGB (1×10^{-5}, 1×10^{-4}, and 5×10^{-4} M). Calculations were made from photographs of the oscilloscope tracings of the progress curve of each reaction. The buffer was the same as that used for the steady-state experiments. Because of the large amounts of plasminogen–streptokinase complex needed for these experiments, assays were carried out only at the above-specified NPGB concentrations.

Steady-State Kinetic Studies. Experiments were performed in a Cary 219 spectrophotometer at 37 °C in 0.1 M potassium phosphate buffer, pH 7.4. Under these conditions, the extinction coefficient of the *p*-nitrophenol at 410 nm is 12 700.

All experiments were carried out in a total volume of 200 μL (both sample and reference cells) in masked microcuvettes.

Experiments differed by the order of addition of reagents to the sample cell. In most cases, plasminogen and streptokinase concentrations were equimolar at 5.0×10^{-6} M with the NPGB concentration at 1×10^{-4} M. In other experiments, NPGB concentrations were varied between 1×10^{-5} and 5×10^{-4} M. Reactions were started by addition of either plasminogen or streptokinase, except for experiments with preformed complexes, to which NPGB was added last. Final values for kinetic constants were averaged from a minimum of 10 experiments.

Equilibrium Binding Studies. Equilibrium binding studies of NPGB to plasminogen were carried out by a gel filtration technique. Samples of plasminogen and NPGB in a total volume of 200 μL were applied to a 0.9 × 13 cm Sephadex G-25C column at room temperature and eluted with 0.1 M potassium phosphate buffer, pH 7.4. One-milliliter fractions were collected, and their absorbance was determined at 402 nm. After addition of 30 μL of 6 N NaOH and mixing, the absorbances at 402 and 280 nm of the completely hydrolyzed NPGB and the plasminogen fraction were determined. Native human and cat plasminogen concentrations were fixed at 8.27×10^{-6} M, and NPGB concentration was varied between 1.0×10^{-5} and 5.0×10^{-3} M by using a stock solution of 10^{-2} M NPGB in dimethylformamide. Upper and lower NPGB concentration limits were found to be imposed on the study experimentally by the solubility and detectability of NPGB and *p*-nitrophenol, respectively. An extinction coefficient of 17 850 at 402 nm was used (Chase & Shaw, 1969). All experiments were repeated at least 3 times.

Results

Routine NPGB titrations of cat plasminogen and streptokinase complexes which were preincubated for 0.5–5 min before addition of NPGB produced bursts (rapid pre-steady-state release of *p*-nitrophenol, equivalent to the enzyme active site concentration) within less than 5 s (Chase & Shaw, 1969; Bender et al., 1967). This behavior is comparable to the one observed in numerous laboratories using human plasmin and human plasminogen–streptokinase complexes (McClintock & Bell, 1971; Reddy & Markus, 1972; Schick & Castellino, 1974; Wohl et al., 1977, 1978, 1979, 1980, 1983). The NPGB reaction thus showed a complete and very rapid acylation of all the active sites in the enzyme complex species, followed by minimal nonspecific NPGB hydrolysis. Preincubated plasminogen–streptokinase titration with NPGB was used solely for quantitation of absolute active site concentrations in each preparation. These values were used for all subsequent determinations of kinetic rate processes. As will be discussed later, these active site concentrations do not change, despite ongoing proteolytic degradation of the enzyme complex (Markus et al., 1976; Wohl et al., 1978).

Preincubated Equimolar Plasminogen–Streptokinase Complex Experiments. Pre-Steady-State Kinetics. Preformed equimolar human and cat plasminogen–streptokinase complexes were mixed with varying concentrations of NPGB (1×10^{-5}–5×10^{-4} M) in the stopped-flow instrument. Optical density values as a function of time were taken from photographs of the progress curves and plotted on a semilog graph in order to derive the *b* values (the first-order rate constants of the pre-steady-state reaction at each given substrate concentration) (Bender et al., 1967; Chase & Shaw, 1969). Their simplified reaction scheme is

$$E + S \overset{K_s}{\rightleftharpoons} E{\cdot}S \xrightarrow{k_2} ES + P_1 \text{ (}p\text{-nitrophenol)}$$

Progress curves photographed for these experiments described pure first-order rates as shown by the semilog plots. There was no indication with either cat or human plasminogen of a complex formation effect. The rationale for this conclusion is that slow complex formation, in addition to the NPGB hydrolysis reaction, would make the process second order and would produce "sigmoid-type" reaction curves. The *b* values derived previously and the corresponding NPGB concentrations were used to make double-reciprocal plots (Chase & Shaw, 1969) and were subjected to linear regression analysis for determination of the rate constants K_s and k_2. These kinetic parameters are summarized in Table I.

Both K_s and k_2 values for the plasminogen–streptokinase complexes are 15-fold higher than those for plasmin, found by Chase & Shaw (1969). Similar, but slightly lower values for plasmin (as compared to our values) were found later, at 30 °C and pH 8.3, by Morris et al. (1981). In this study, the k_2 values were close to those for trypsin (1.95 s^{-1}), while the K_s values were 20-fold higher than those for plasmin (11.3 μM). We have found previously with H-D-Val-Leu-Lys-pNA that the plasminogen–streptokinase complex has higher K_m and k_{cat} values for both ester and amide substrates than does plasmin (Wohl et al., 1980). These results are not surprising in view of the high specificity of the plasminogen–streptokinase complex for its plasminogen substrate, but not for small substrates.

Table I: Kinetic Parameters of the Interaction of Substrates with Cat and Human Plasminogens and Their Streptokinase Complexes

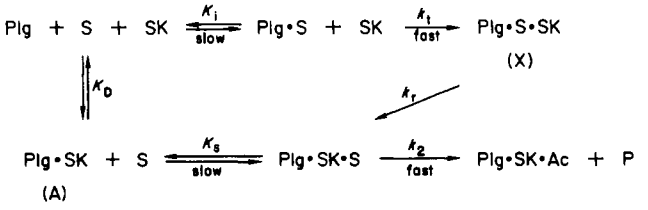
method and species	substrate or inhibitor	kinetic parameters			
		K_s (μM)	k_2 (s^{-1})	$t_{1/2}$ (s)	K_i (μM)
pre-steady-state data[a]					
cat Plg·SK	NPGB	320 + 35	5.50 ± 0.60	0.126	
human Plg·SK		200 ± 25	6.67 ± 0.60	0.104	
steady-state data[b]			k_r[b]		
cat Plg + SK[c]	NPGB		0.010 ± 0.002	69.30 ± 5.86	2.1
	H-D-Val-Leu-Lys-pNA				1550
	benzamidine				6900
human Plg + SK[c]	NPGB		0.100 ± 0.010	7.54 ± 0.75	2660
	H-D-Val-Leu-Lys-pNA				1700
	benzamidine				7800

[a] These experiments were carried out with preformed (mol/mol) plasminogen–streptokinase complex preparations at pH 7.4 and 37 °C (see Materials and Methods). [b] Steady-state kinetics were carried out with preformed (mol/mol) plasminogen–streptokinase complex preparations for obtaining benzamidine data, but for NPGB and H-D-Val-Leu-Lys-pNA experiments, the reaction was started by addition of streptokinase to a cuvette which contained all the other reagents. All experiments were performed with a total reaction volume of 200 μL; k_r is the first-order rate constant of the *p*-nitrophenol production in these experiments (see text). [c] NPGB binding studies were carried out in the absence of streptokinase; experimental details are described in the text.

Steady-State Kinetic Experiments. The preincubation time of both human and cat plasminogens with equimolar streptokinase was varied from 0.5 min (close to the minimum mixing time) to 5 min. In all experiments, regardless of incubation time, addition of NPGB produced an immediate full burst with identical A_{410nm} values (equivalent to the concentration of active sites).

We carried out a series of experiments varying the concentration of NPGB, plasminogen, and streptokinase, while adding either plasminogen or streptokinase last to the reaction mixture. The results of these experiments showed that the order of addition influenced strongly the kinetic behavior of the system (see Figure 1). Addition of streptokinase last resulted in first-order progress curves much slower than those resulting from the use of premixed plasminogen–streptokinase complex. Addition of plasminogen last resulted in a partial burst and then a slow first-order reaction. These results suggested that NPGB interferes with the formation of an active site in the plasminogen–streptokinase complex.

Comparison of the results of the preformed plasminogen–streptokinase NPGB titrations with those of the streptokinase added last data points clearly to an inhibition phenomenon. Since the only possible inhibitory component in these experiments is NPGB, it is evidently binding to one of the component proteins. By necessity, the only enzyme species in the system which can hydrolyze NPGB is the plasminogen–streptokinase complex. Further, streptokinase could bind to the plasminogen–NPGB complex, but this ternary complex is definitely different from the one obtained by adding the substrate to the plasminogen–streptokinase complex (A). While the latter is a fully active enzyme–inhibitor complex, the former (X) develops an active site slowly, which consequently hydrolyzes the bound NPGB molecule. A reaction scheme with a simple kinetic mechanism that can account for the previous observations is proposed below:



The essential features of this mechanism are that k_t has to be much faster than the rearrangement of the plasminogen–S–streptokinase complex ($k_t \gg k_r$) and that k_2 is much faster than the dissociation of the plasminogen–streptokinase complex

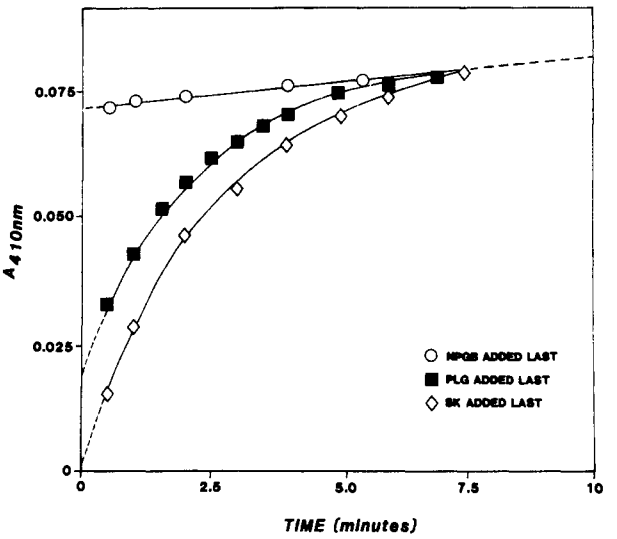


FIGURE 1: Active site titration of the cat plasminogen–streptokinase complex with NPGB. The titrations were performed in a microcuvette with a total volume of 200 μL at pH 7.4 and 37 °C (see Materials and Methods) with plasminogen and streptokinase concentrations at 7.5×10^{-6} M and NPGB concentration at 1×10^{-4} M. Experiments varied by the order of addition of the last reagent: NPGB last with a 0.5-min preincubation of the plasminogen–streptokinase complex (○); plasminogen last (■); streptokinase last (◇).

($k_2 \gg k_s$). If indeed these conditions are satisfied, then by varying the order of addition of the reagents, one can determine k_2 and k_r experimentally.

NPGB Titration of Equimolar Plasminogen–Streptokinase When Streptokinase Is Added Last. In these experiments, the NPGB concentration was varied between 1×10^{-5} and 5×10^{-4} M, while the plasminogen concentration was equal to the streptokinase concentration, 5.0×10^{-6} M. The rate of the reaction was 550-fold slower with cat plasminogen when streptokinase was added last (complex X) than the rate of the same components with NPGB added last (complex A). The reaction curve could be extrapolated back to zero time and obeyed pure first-order kinetics. Most importantly, the experimental first-order rate constants were independent of the concentration of NPGB (see Table II). The same behavior was also observed for human plasminogen, except that the rate of the reaction was only 66-fold slower than that of the corresponding preformed complex (A). NPGB was not hydrolyzed by either plasminogen alone or streptokinase alone, and final titration values were in each case equivalent to 100% of the potential active site concentration. It was also true that

Table II: First-Order Rate Constants of Acylation of Cat Plasminogen–Streptokinase Complex with Varying NPGB Concentration and with Streptokinase Added Last[a]

[NPGB] (μM)	k_r (s^{-1})	[NPGB] (μM)	k_r (s^{-1})
10	0.010	150	0.012
25	0.009	250	0.011
50	0.008	500	0.009

[a] These experiments were performed at equimolar concentrations of plasminogen and streptokinase of 5.0×10^{-6} M under conditions described in the text.

Table III: Varying NPGB Concentration with Constant and Equimolar Concentrations of Cat Plasminogen and Streptokinase and with Plasminogen Added Last[a]

[NPGB] (M)	Π_0 (A_{410})	$[\ln [\Pi_\infty/(\Pi_\infty - \Pi_0)]]/(\Pi_\infty - \Pi_0)$[b] (value $\times 10^3$)
5×10^{-5}	0.050	97.7
1×10^{-4}	0.043	49.2
2.5×10^{-4}	0.035	25.7
5.0×10^{-4}	0.025	12.1

[a] See text for experimental details. [b] Experimental values used to calculate the ratio of rates of plasminogen–streptokinase and plasminogen–NPGB complex formation with varying NPGB concentrations; the equations are detailed in the text.

despite the appreciably slower reaction rate, we still could not detect the binding of streptokinase to plasminogen. Indeed, if the latter reaction had been slow, on our experimental time scale, the release of *p*-nitrophenol should have shown an increasing initial slope which indicated increasing rates of hydrolysis of NPGB and streptokinase complex formation. On the other hand, if the formation of the plasminogen–S–streptokinase complex (X) were the rate-limiting step, then the release of *p*-nitrophenol should also have obeyed second-order kinetics. Thus, according to the reaction scheme shown, k_r must be the rate-limiting step.

To investigate whether streptokinase binds NPGB, streptokinase concentration was varied from 2.5×10^{-6} to 3.0×10^{-5} M (12-fold), while plasminogen concentration was held constant at 5.0×10^{-6} M and NPGB concentration was 1×10^{-5} M. The results showed the first-order rate constant to be independent of streptokinase concentration, and consequently that streptokinase does not bind NPGB. Neither plasminogen nor streptokinase hydrolyzes NPGB, despite the similarity of the primary sequence of streptokinase to that of serine proteases, as reported by Jackson & Tang (1982). As can be deduced from the results shown in Figure 1, k_r is independent of the order of addition of the reagents.

NPGB Titration Experiments with Plasminogen Added Last. When plasminogen was added last to the reaction mixture, equimolar plasminogen and streptokinase concentrations were 5.0×10^{-6} M, and the NPGB concentration was 1×10^{-5} M, the reaction curve described a burst plus a first-order reaction (see Figure 1) to account for the total active site concentration (a quantity of *p*-nitrophenol only released by the end of the slow phase of the reaction). As the NPGB concentration increased, the size of the burst decreased. In another experiment, the NPGB concentration was constant at 1×10^{-5} M, and the streptokinase concentration was 5.0×10^{-6} M, while the plasminogen concentration was varied from streptokinase equivalence to 4-fold molar concentration. As before, the size of the burst and the first-order rate constant decreased from 80% to a few percent (Table III).

From the reaction scheme described previously and the data in Table II, we can derive the kinetic expression for the ratio of the rate of formation of cat plasminogen–streptokinase–S

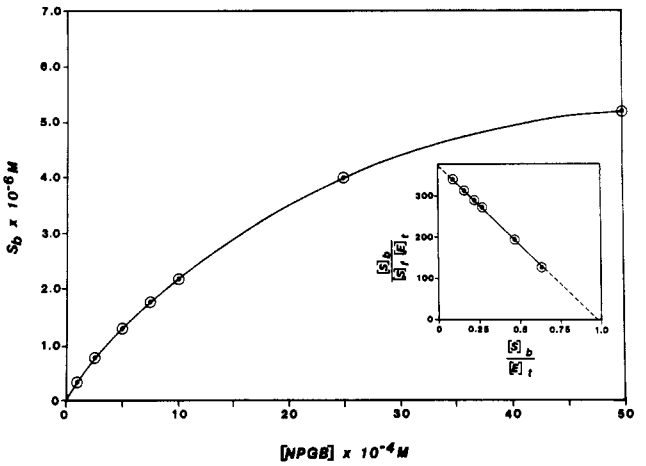


FIGURE 2: Binding curve and Scatchard plot (inset) of the binding of NPGB to native human plasminogen at room temperature. Each experimental point was calculated from the concentration of NPGB present in the plasminogen peak eluate of a 0.9 × 13 cm G-25C column. The experimental conditions are detailed under Materials and Methods.

complex to plasminogen–NPGB–streptokinase complex when plasminogen is added last. This ratio (k_D/k_i) is derived from the ratio of the equilibrium equations indicated in the previous reaction scheme. The concentrations of each reactant were converted to absolute quantities as they were measured by the NPGB titration. In this case, $[Plg]_0 = [SK]_0$, NPGB concentration was varied, and plasminogen was added last to the reaction mixture. The appropriate final equation is

$$k_D/k_i = [NPGB]\frac{\ln ([SK]_0/[SK]_0 - [Plg{\cdot}SK])}{[SK]_0 - [Plg{\cdot}SK]}$$

which is equal to

$$k_D/k_i = [NPGB]\frac{\ln [\Pi_\infty/(\Pi_\infty - \Pi_0)]}{\Pi_\infty - \Pi_0}$$

where Π_∞ is equal to the full burst ($=[SK]_0 = [PLG]_0$) and Π_0 is the partial burst, which is equal to the "instantaneous" plasminogen–streptokinase complex formed [for a complete discussion of the theory of these considerations, see Bender et al. (1967) and Chase & Shaw (1969)] (k_D and k_i were defined in the reaction scheme shown previously in this paper). The ratio of k_D/k_i obtained from the data in Table III is 7.8. This ratio of k_D/k_i suggested that binding of NPGB to cat plasminogen is indeed very strong and probably that the plasminogen–streptokinase dissociation constant is much higher than that of the human plasminogen complex. Furthermore, the results indicate that indeed only plasminogen binds NPGB and that the binding of NPGB to plasminogen possibly interferes with the plasminogen–streptokinase activator complex formation.

Studies of NPGB Binding to Plasminogen. The results of NPGB binding experiments with native human plasminogen were analyzed, and the data are shown in Figure 2. Values for the dissociation constants for both human and cat plasminogens are summarized in Table I. Each value and each data point were averaged from a minimum of three experiments. The value of K_i for native cat plasminogen and NPGB was not derived from a full saturation curve, but only from experiments with 75% and higher saturating concentrations. This was necessitated by the low K_i and the limit of sensitivity of detection of *p*-nitrophenol. At 5×10^{-5} M NPGB concentration, cat plasminogen is completely saturated. Three other important aspects of these reactions were learned from the binding experiments. The first was the fact that indeed

we are observing an ester binding to plasminogen, rather than an event of acylation. This fact was proven by the absence of *p*-nitrophenol absorbance before hydrolysis with NaOH. The second observation was that the break between the protein peak and the NPGB peak in the eluates was not complete. This showed that as the plasminogen–NPGB complex was passing through the Sephadex G-25 column it tended to dissociate, proving that plasminogen and NPGB are in equilibrium with their complex. The third important finding was that within experimental error, both native human and cat plasminogens bind one ligand molecule at saturating ligand concentrations. This fact was found to be true for cat plasminogen, even at 1000-fold K_i ligand concentrations. For human plasminogen, only an extrapolation of the data is available (Figure 2).

Competition of Substrates and Inhibitors for the Plasminogen Binding Site. On the basis of the results of the plasminogen–ligand binding experiments, it became obvious that we could explain all the data on the basis of two different pathways which would still follow the primary mechanism proposed above. The first mechanism involves binding of ligand to plasminogen at a site other than the active site and preventing consequent normal binding of streptokinase to plasminogen, resulting in abnormal rates of active site formation. In this case, streptokinase will still bind to plasminogen, but the ligand may or may not be able to dissociate from the ternary complex. The active site, when formed, would bind and hydrolyze free substrate molecules which would stay in equilibrium with the rest of the substrate population. In the case of the second mechanism, the NPGB molecule, already tightly bound to the active site on plasminogen, becomes trapped when streptokinase binds to the complex, and is then hydrolyzed. During this process, the trapped substrate is no longer in equilibrium with free substrate in the bulk solution; i.e., it cannot exchange with competitive inhibitors.

To distinguish between these two possible mechanisms, the following experiment was performed. Cat plasminogen was added to a mixture of NPGB and streptokinase in buffer, as before. After the initial burst, a high concentration (0.1 M final concentration) of benzamidine was added and the reaction allowed to continue. The first-order progress curve in this experiment was superimposable on that of the control experiment (no benzamidine) (Figure 1). In addition, when the plasminogen was preincubated with a high concentration of benzamidine and then diluted into the streptokinase–NPGB solution, it exhibited no burst but rather a slow first-order progress curve, proving that benzamidine and NPGB are indeed competing for the same binding site.

To explore further the results discussed so far, competitive inhibition experiments were performed with H-D-Val-Leu-Lys-pNA (see Table I) which competed directly with NPGB. Similarly, ϵ-aminocaproic acid concentrations were varied, and it became clear that unless 0.1 M or higher concentrations were used, this ligand, which is known to affect plasminogen conformation, did not affect NPGB binding at all.

The plasmin substrate (H-D-Val-Leu-Lys-pNA) experiments proved that the delay in activation of cat plasminogen by catalytic concentrations of streptokinase (Wohl et al., 1983) is not due to substrate inhibition but to a slow formation of the plasminogen–streptokinase activator complex. This explains why we observed non-Michaelis–Menten kinetics when cat plasminogen was activated with streptokinase (Wohl et al., 1983). The absence of sigmoid kinetics in our system with human plasminogen is explained by the very high K_i of H-D-Val-Leu-Lys-pNA (10 times higher than the substrate concentration in our activation system).

Discussion

Studies of binding and hydrolysis of NPGB by zymogens of serine proteases are not unique to this report. Such interactions have been well characterized for trypsinogen and chymotrypsinogen (Robinson et al., 1973; Gertler et al., 1974; Kerr et al., 1975). However, the strength of the binding and the absence of any hydrolytic activity are unique to plasminogen. Teleologically, the complete lack of an active site is a necessary prerequisite for zymogens of blood proteases.

A summary of the basic conclusions that can be drawn from the results of this study follows: (1) NPGB and other low molecular weight active site directed compounds bind specifically to the potential substrate binding site of plasminogen (both cat and human). (2) Such binding does not prevent streptokinase binding; rather, a ternary complex is formed, which is different from that generated by the addition of the substrate to the plasminogen–streptokinase complex. This new ternary complex rearranges slowly to the active plasminogen–streptokinase–substrate (inhibitor) complex (k_r). (3) Formation of the active site in the plasminogen–streptokinase complex is prevented temporarily by the presence of a small ligand in the potential binding pocket of plasminogen; consequently, this structure, as well as the rest of the active site, is involved directly in streptokinase binding. (4) The benzamidine experiments show that addition of streptokinase to the plasminogen–S complex traps the ligand in the plasminogen binding site and prevents it from exchanging with other molecules in the solution until development of the active site, via structural rearrangement, is complete; this rearrangement proceeds with pure first-order kinetics. (5) All evidence indicates that once formed, the acylated plasminogen–streptokinase complex is the same whether it was formed by the pathway leading to complex A or the pathway involving complex X. In fact, when both enzymes are deacylated, they both have the same activity toward the tripeptide substrate. (6) Since activation of plasminogen by urokinase is not affected by small substrate binding to plasminogen, it is likely that the urokinase–plasminogen (E·S) complex must involve binding only in the immediate neighborhood of the scissile bond, whereas streptokinase binding involves both the scissile bond and the active site structures, as we postulated previously (Wohl et al., 1977, 1978, 1979, 1980) and as shown in this study; data to support this statement have been reported previously (Wohl et al., 1983). (7) The results described in this study are common to human and cat plasminogens and are probably common to all other plasminogen species. Differences between plasminogen species are manifested through different rate constants, and that is why the effects were not obvious with human plasminogen but were very much so with cat plasminogen (10-fold difference in rates).

The mechanism we have proposed under Results was found to be the most parsimonious conceptual consideration of the data. This mechanism takes into consideration only the slow reactions in this system, i.e., those that can be observed by steady-state kinetic methods. It is possible, and actually probable, that a conformational change accompanies the transition of complex X to A·S. Such a change had been found in the trypsinogen to trypsin transition by X-ray crystallography (Bode & Huber, 1976; Bode et al., 1978). We do not have data to show such a conformational change in plasminogen, and by itself, such a transition cannot account for the data observed in this study.

Theoretically, activation of trypsinogen or chymotrypsinogen could occur in a manner similar to that of equimolar plas-

minogen activation by streptokinase, but the fact that this is not known to happen makes the latter mechanism unique. We can postulate on the basis of this unique difference and the findings in these studies that the potential active site in plasminogen is much less distorted than in other serine protease zymogens (as proved by the presence of a strong binding site) and that it probably only needs the removal of a small obstruction to become fully operational. Such an effect is achieved upon binding of streptokinase. Similarly, the specificity of plasminogen activation is then mostly attributable to properties lent to the activator complex by streptokinase rather than the plasminogen active site.

On the basis of these studies, it is also reasonable to postulate that similar interactions are possible in vivo with small or large protein or nonprotein ligands. It is possible that the strong lysine binding site on plasminogen which is thought to be a universal fibrinolytic pathway regulatory site (Lijnen et al., 1980) may not be the only regulatory site on plasminogen. Such studies will be worth pursuing.

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Registry No. NPGB, 21658-26-4; H-D-Val-Leu-Lys-pNA, 63589-93-5; benzamide, 55-21-0.

References

Bender, M. L., Kézdy, F. J., & Wedler, F. C. (1967) *J. Chem. Educ. 44*, 84–88.

Bode, W., & Huber, R. (1976) *FEBS Lett. 68*, 231–236.

Bode, W., Schwager, P., & Huber, R. (1978) *J. Mol. Biol. 118*, 99–112.

Chase, T., Jr., & Shaw, E. (1969) *Biochemistry 8*, 2212–2224.

Friberger, P., Knös, M., Gustavsson, S., Aurell, L., & Claeson, G. (1978) *Haemostasis 7*, 138–145.

Gertler, A., Walsh, K. A., & Neurath, H. (1974) *Biochemistry 13*, 1302–1310.

Jackson, K. W., & Tang, J. (1982) *Biochemistry 21*, 6620–6625.

Kerr, M. A., Walsh, K. A., & Neurath, H. (1975) *Biochemistry 14*, 5088–5094.

Lijnen, H. R., Hoylaerts, M., & Collen, D. (1980) *J. Biol. Chem. 255*, 10214–10222.

Markus, G., Evers, J. L., & Hobika, G. H. (1976) *J. Biol. Chem. 251*, 6495–6504.

McClintock, D. K., & Bell, P. H. (1971) *Biochem. Biophys. Res. Commun. 43*, 694–702.

Morris, J. P., Blatt, S., Powell, J. R., Strickland, D. K., & Castellino, F. J. (1981) *Biochemistry 20*, 4811–4816.

Reddy, K. N. N., & Markus, G. (1972) *J. Biol. Chem. 247*, 1683–1691.

Robbins, K. C., & Summaria, L. (1970) *Methods Enzymol. 19*, 184–199.

Robinson, N. C., Neurath, H., & Walsh, K. A. (1973) *Biochemistry 12*, 420–426.

Schick, L. A., & Castellino, F. J. (1974) *Biochem. Biophys. Res. Commun. 57*, 47–54.

Segel, I. H. (1976) in *Biochemical Calculations*, 2nd ed., p 243, Wiley, New York.

Summaria, L., Wohl, R. C., Boreisha, I. G., & Robbins, K. C. (1982) *Biochemistry 21*, 2056–2059.

Wohl, R. C., Arzadon, L., Summaria, L., & Robbins, K. C. (1977) *J. Biol. Chem. 252*, 1141–1147.

Wohl, R. C., Summaria, L., & Robbins, K. C. (1978) *J. Biol. Chem. 253*, 1402–1407.

Wohl, R. C., Summaria, L., & Robbins, K. C. (1979) *J. Biol. Chem. 254*, 9063–9069.

Wohl, R. C., Summaria, L., & Robbins, K. C. (1980) *J. Biol. Chem. 255*, 2005–2013.

Wohl, R. C., Sinio, L., Summaria, L., & Robbins, K. C. (1983) *Biochim. Biophys. Acta 745*, 20–31.