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MECHANISM OF REACTION OF HUMAN PLASMIN WITH α-*N*-BENZOYL-L-ARGININE-*p*-NITROANILIDE

TITRATION OF THE ENZYME

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SUMMARY

Human plasmin (EC 3.4.4.14) a key enzyme in fibrinolysis which possesses a trypsin-like specificity catalyses the hydrolysis of α-*N*-benzoyl-L-arginine-*p*-nitroanilide (Bz-L-Arg-OpNAn). The reaction was investigated in the steady state by the usual initial-velocity studies and in the presteady state by the stopped-flow method. The results were compatible with the three-step mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_2} ES_2 + P_1,\ ES_2 \xrightarrow{k_3} E + P_2$$

where P_1 is *p*-nitroaniline and P_2 is α-*N*-benzoyl-L-arginine, thus supporting the theory of the formation of an acyl–enzyme intermediate in normal plasmin catalysed reactions.

The concentration of the active sites of plasmin as determined from the "burst" of *p*-nitroaniline and from titration with polyvalent protease inhibitor was in good agreement.

The kinetic constants (Tris–HCl, 0.15 mol/l, pH 7.8, 25 °C) were: $k_1 > 10^5$ $l \cdot mol^{-1} \cdot s^{-1}$, $k_{-1} > 10^3\ s^{-1}$, $k_2 = 16\ s^{-1}$, $k_3 = 0.09\ s^{-1}$, $k_{cat} = k_2k_3/(k_2 + k_3) = 0.09\ s^{-1}$, $K_s = (k_{-1} + k_2)/k_1 = 12$ mmol·l^{-1}, $K_m = K_sk_3/(k_2 + k_3) = 60\ \mu mol \cdot l^{-1}$.

INTRODUCTION

Plasmin (EC 3.4.4.14) is a serine protease originating from an inactive precursor, plasminogen, which is present in animal blood plasma. Physiologically it has a very specific and selective function, i.e. the degradation and dissolution of fibrin deposits in the blood vessels.

Abbreviations: Tris, trishydroxymethyl aminomethane. Bz-L-Arg-OpNAn, α-*N*-benzoyl-L-arginine-*p*-nitroanilide. Bz-L-Arg-OEt, α-*N*-Benzoyl-L-arginine ethyl ester.

Different investigations have shown that reactions catalysed by trypsin can be analysed in terms of Eqn 1 [1].

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_2} ES_2 + P_1,\ ES_2 \xrightarrow{k_3} E + P_2 \tag{1}$$

A similar mechanism has been proposed for plasmin [2, 3].

The most important experimental evidence for this is a relatively fast pre-steady-state release of *p*-nitrophenol occurring in the plasmin-catalysed hydrolysis of *p*-nitrophenyl-*p'*-guanidinobenzoate [2, 3], and *N*-(*p*-carboxybenzyl) pyridinium-*p*-nitrophenyl ester bromide [3]. These substrates show very low turnover numbers, k_{cat}. The *p*-nitrophenyl-*p'*-guanidinobenzoate value for k_{cat} was determined [2] to be $5.2 \cdot 10^{-6}\ s^{-1}$, the *N*-(*p*-carboxybenzyl) pyridinium-*p*-nitrophenyl ester bromide value was claimed too low to be measured [3]. The Michaelis constants K_s ($= (k_{-1} + k_2)/k_1$) were low too, as compared with the K_s values for different trypsin substrates [4, 5]. These compounds thus act more like strong inhibitors than as substrates, and consequently the reaction mechanisms may be different from the normal mechanism for plasmin-catalysed reactions. The substrate used in the present study, *α*-*N*-benzoyl-L-arginine-*p*-nitroanilide (Bz-L-Arg-OpNAn) resembles the natural substrates of plasmin in being a *N*-substituted amide of arginine, wherefore a study with this substrate may give more relevant information on the normal plasmin reaction.

Titration procedures have advantages over rate assays for the assay of the concentration of enzymes. One method of titration is based on presteady-state kinetics. Here the amount of the product P_1 formed in the presteady-state period under certain conditions, is taken as a measure of the enzyme concentration [6]. Another method consists of the establishment of the amount of substance of a strong inhibitor necessary to inhibit completely the enzymatic activity of the enzyme. This method has been employed for the assay of the concentration of plasmin by Ganrot [7].

The present work has two objects. First to investigate whether the kinetics of the presteady-state and the steady-state reactions of the hydrolysis of Bz-L-Arg-OpNAn with plasmin are satisfactorily described by Eqn 1. The aim was to describe the different steps of the reaction by evaluating the individual rate constants. The second object was to compare the values obtained by the two methods quoted for the assay of the active site concentration of plasmin.

THEORY

Steady-state kinetics

The reaction mechanism of Eqn 1 leads to the steady-state rate equation

$$v = \frac{V}{1 + \dfrac{K_m}{s_0}} = \frac{k_{cat} e}{1 + \dfrac{K_m}{s_0}} \tag{2}$$

s_0 = initial substrate concentration, e = total enzyme (active site) concentration, V = maximum velocity, $k_{cat} = k_2 k_3/(k_2 + k_3)$, the apparent Michaelis constant $K_m = K_s k_3/(k_2 + k_3)$, and the Michaelis constant $K_s = (k_{-1} + k_2)/k_1$. Ouellet and Stewart [6] uses $K_m \triangleq K^a$ and $K'_m \triangleq K_m$.

Values for both K_m and V can be obtained from a plot of substrate concentration divided by initial velocity versus substrate concentration (Hanes plot) [8].

$$\frac{s_0}{v} = \frac{1}{V} s_0 + \frac{K_m}{V} \tag{3}$$

In the presence of a linear competitive inhibitor [9], K_m becomes

$$K_{m(i)} = K_m (1 + i/K_i) \tag{4}$$

i = inhibitor concentration, K_i = dissociation constant of the enzyme–inhibitor complex.

Presteady-state kinetics

The theory of the presteady-state kinetics of the reaction mechanism described by Eqn 1 is given by Ouellet and Stewart [6]. The quoted paper contains the detailed derivation of equations and definitions of constants used below.

In the theory the concentration of product P_1 as a function of time shows an initial exponential rise followed by a second exponential part with a decreasing rate approaching the steady state.

$$P_1 = vt + A(e^{Ft} - 1) + B(e^{Gt} - 1) \tag{5}$$

Here v is the steady-state velocity (Eqn 2). A, B, F and G are constants. $A = k_2M/F$ and $B = k_2N/G$ in the notation of Ouellet and Stewart [6].

When t becomes large we get the steady-state production of *p*-nitroaniline

$$P_{1s} = vt - A - B = vt + \pi \tag{6}$$

Subtracting Eqn 5 from Eqn 6, setting $e^{Gt} \approx 0$ (ref. 6) we get

$$P_{1s} - P_1 = -Ae^{Ft} \text{ or } \ln (P_{1s} - P_1) = Ft + C \tag{7}$$

$(P_{1s} - P_1)$ is determined as the difference between the concentration of *p*-nitroaniline calculated by extrapolation of the steady-state line to the time of observation and the actual concentration of the product formed, or any quantity proportional to these concentrations.

Under the assumption that $k_3 \ll k_2$

$$F = -k_2 (1 + K_m/s_0) (1 + K_s/s_0)^{-1} \tag{8}$$

If F is determined as a function of the substrate concentration (s_0) the plot of $-(1 + K_m/s_0)/F$ versus s_0^{-1} would give $1/k_2$ as the intercept and $K_s/k_2 = K_m/k_{cat}$ as the slope. Obtaining K_m from the steady-state plot (Eqn 3), k_{cat} can be calculated. Furthermore k_3 can be obtained from $k_3 = k_2k_{cat}/(k_2 - k_{cat})$. Thus it is possible to control the assumption $k_2 \gg k_3$.

When steady-state prevails a straight line is obtained (Eqn 6). The "burst"

size, π, is the intercept obtained on the P_1 axis by extrapolating the steady-state line to the zero line. When substrate concentration greatly exceeds enzyme (active site) concentration and no inhibitors are present [6], then

$$\pi = k_{cat}^2 \, e \, (1 + K_m/s_0)^{-2} \, (k_3^{-2} - (k_1 k_2 s_0))^{-1} \tag{9}$$

Thus π becomes positive only when $k_3^2 < k_1 k_2 s_0$. It is reasonable to assume that $k_3^2 \ll k_1 k_2 s_0$, as k_1 is usually very large. Then

$$\pi = k_{cat}^2 \, e \, (1 + K_m/s_0)^{-2} \, k_3^{-2} \tag{10}$$

$$e = \pi \, k_3^2 \, (1 + K_m/s_0)^2 \, k_{cat}^{-2} \rightarrow \pi \, (1 + K_m/s_0)^2 \text{ when } k_2 \gg k_3 \tag{11}$$

The value of e thus calculated can be compared to values calculated from titration with a strong inhibitor.

At the very beginning of the reaction the initial exponential has a retarding effect on the appearance of the product P_1. This short induction time τ [6] is

$$\tau = \frac{1}{k_1(s_0 + K_s)} \tag{12}$$

when τ is $< h$, $1/k_1 < h \, (s_0 + K_s)$

and

$$k_1 > \frac{1}{h(s_0 + K_s)} \tag{13}$$

and

$$k_{-1} = k_1 K_s - k_2 > \frac{K_s}{h(s_0 + K_s)} - k_2 \tag{14}$$

Thus, even if the small exponential is undetectable, it is possible to obtain minimal values for k_1 and k_{-1}.

If it is verified that $k_{-1} \gg k_2$

$$K_s \approx \frac{k_{-1}}{k_1} \tag{15}$$

Statistical symbols

The quantities given in the tables are the best estimate based on regression analysis and the standard error of the estimate, symbolized by: $y \pm s_y$. Consequently the observed values fall within $y \pm 2s_y$ with $P = 0.95$.

MATERIALS

Human plasminogen was prepared by affinity chromatography on a lysine-substituted Sepharose column as described by Deutsch and Mertz [10]. It was kept in a lyophilized state at −20 °C. The preparation contained protein $m = 149$ mg, disodiumhydrogenphosphate $n = 0.30$ mmol, and potassium dihydrogenphosphate $n = 0.08$ mmol.

Human plasmin was prepared by maximal activation of human plasminogen by affinity chromatography on urokinase-substituted Sepharose (Müllertz, S. and Andersen, I., unpublished). Plasmin (mass concentration, 2.0 g/l) was stored at −20 °C in buffer (phosphate, 5 mmol/l; lysine, 0.1 mol/l, pH 7.4). Under these conditions plasmin was stable for several months.

The plasminogen and plasmin preparations were found to contain only small amounts of contaminating proteins.

Polyvalent protease inhibitor from bovine lung (Trasylol®) was purchased from Bayer, Leverkusen, West Germany. $5 \cdot 10^3$ KIE/l dissolved in NaCl, 0.15 mol/l.

Crystalline bovine trypsin and crystalline soybean trypsin inhibitor was purchased from NOVO A/S, Copenhagen.

Substrates

α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide (Bz-DL-Arg-OpNAn) and α-*N*-benzoyl-L-arginine ethyl ester (Bz-L-Arg-OEt) was obtained from Sigma Chemical Company, U.S.A.

α-*N*-benzoyl-D-arginine-*p*-nitroanilide (Bz-D-Arg-OpNAn) was prepared according to Erlanger et al. [11]. As pure Bz-L-Arg-OpNAn was not available, the experiments had to be performed in the presence of Bz-D-Arg-OpNAn, the concentration of which was kept constant. In all kinetic experiments the solvent used was Tris–HCl buffer, 0.15 mol/l, pH 7.8, $I = 0.15$, pH 7.8 (Tris).

METHODS

Bz-L-Arg-OEtesterase activity was determined as described by Worning and Müllertz [12].

The concentration of active sites of plasmin and trypsin was determined by titration with the polyvalent protease inhibitor according to Ganrot [7], but using the Bz-L-Arg-OEtesterase assay throughout.

Steady-state kinetics

The formation of *p*-nitroaniline by the hydrolysis of Bz-L-Arg-OpNAn was measured by following the change in absorbance at $\lambda = 410$ nm. The molar absorbance coefficient (ε) of *p*-nitroaniline at this wavelength is $8800\ l \cdot mol^{-1} \cdot cm^{-1}$ [11]. The change in absorbance was measured every 30 s for 15–30 min in a Zeiss PMQ II spectrophotometer thermostated at 25 °C. Cuvettes with a length of lightpath of 10 mm were used and the total volume of the reaction mixture was 1.5 ml. The enzymatic reaction was started by the addition of 100 μl of a plasmin solution containing 201 μg of protein.

All assays were performed in duplicate and some in triplicate. The initial-velocity data were correlated with a standard plasmin Bz-L-Arg-OEt assay that was conducted daily in order to correct for possible minor day to day variations, and as a control of the enzyme stock solution.

Progress curves were obtained by plotting the concentration of *p*-nitroaniline as a function of time. Initial velocities were taken as the slope of the first part of the progress curves, which was linear in all cases.

Presteady-state kinetics

The presteady-state progress of the hydrolysis of Bz-L-Arg-OpNAn was measured with a Durrum–Gibson stopped-flow spectrophotometer [13], equipped with a cuvette with a path-length of 20 mm. All measurements were made at $\lambda = 410$ nm. The reaction system was thermostated at 25 °C. All solutions were made up in gas-free water. Prior to a typical run the oscilloscope was calibrated for transmittance ($T = 1$) with substrate solution and buffer solution in the drive syringes. The buffer solution was then replaced by enzyme solution and approx. 200 μl of each of the solutions were injected into the cuvette. At least 10 min of thermostating time was allowed each time after the drive syringes had been filled with reactants. After three or four traces reflecting the rate of hydrolysis of Bz-L-Arg-OpNAn had been reproduced on the oscilloscope a polaroid photograph was taken. Data were obtained from enlargements of the oscilloscope pictures. The y and x coordinates of 10 points of the apparently linear late part of the curve were read from the enlargement. In accordance with the principle of least squares the linear regression (Eqn 6), representing the extrapolated steady-state rate, the intercept π and the standard error of the estimate of π were calculated. Furthermore, the y and x coordinates of 10 points of the initial exponential curve were read from the enlargement. The constant F was determined from the distances between the points read from the initial exponential curve and equivalent points on the straight line representing the extrapolated steady-state rate (Eqn 7).

RESULTS

Steady-state kinetics

Initial velocities were measured at six concentrations of Bz-L-Arg-OpNAn varying from 0.02–0.8 mmol/l at three different fixed concentrations of Bz-D-Arg-OpNAn varying from 0.8–2.4 mmol/l. This was the maximal range of Bz-D-Arg-OpNAn attainable, at the lower limit because of the inherent content of Bz-D-Arg-OpNAn in the substrate, and at the upper limit because of the solubility.

Initial velocities were read from the curves and multiplied by the dilution factor to make them equivalent to the enzyme concentration of the stock solution. The regression of s_0/v on s_0 (Eqn 3) was computed according to the method of least squares.

It was found that the Michaelis–Menten relationship was obeyed. A Hanes plot is shown in Fig. 1.

To determine whether Bz-D-Arg-OpNAn was an inhibitor or not, three different fits were made, one for each fixed Bz-D-Arg-OpNAn concentration. The maximal veloxity V, was taken as the reciprocal of the slope. The apparent Michaelis constant, K_m, was taken as the intercept on the horizontal axis multiplied by V. Table I shows the collected data, both the apparent K_m values ($K_{m(i)}$) (Eqn 4) and the K_m values after correction for the influence of the Bz-D-Arg-OpNAn (K_m) as well as the maximal velocities (V) as referring to the plasmin concentration of the stock solution.

V showed no significant variation, while K_m was possibly significantly ($0.1 > P > 0.05$) larger at Bz-D-Arg-OpNAn 2.4 mmol/l than at 0.8 mmol/l. Bz-D-Arg-OpNAn may thus be a weak competitive inhibitor. A calculation of K_i according to Eqn 4 gave $K_i = 17.7$ mmol.l^{-1}.

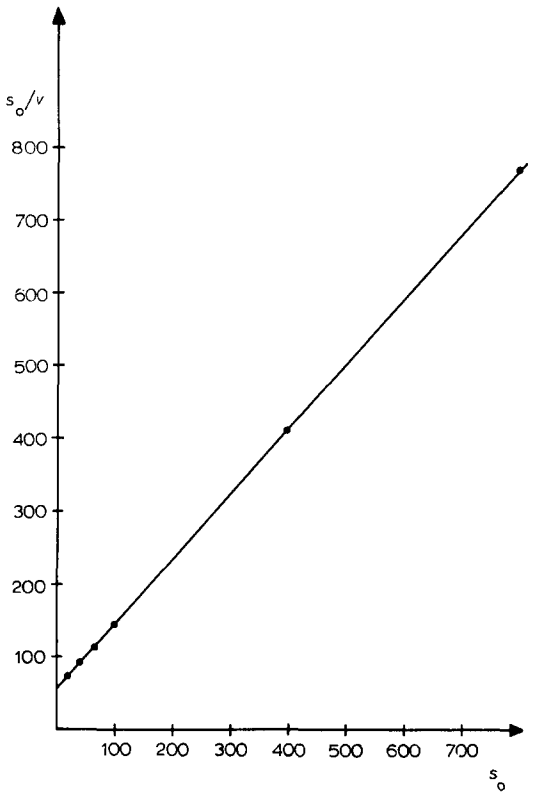

Fig. 1. Hanes plot (Eqn 3) of the plasmin-catalysed hydrolysis of Bz-L-Arg-OpNAn at a concentration Bz-D-Arg-OpNAn of 0.8 mmol·l^{-1}. Ordinate (s_0/v), the concentration of Bz-L-Arg-OpNAn divided by the initial velocity of the reaction as calculated for the enzyme stock solution/s; abscissa (s_0), the concentration of Bz-L-Arg-OpNAn/μmol·l^{-1}.

Presteady-state kinetics

Experiments were conducted at five concentrations of Bz-L-Arg-OpNAn varying from c (final) = 0.05–0.25 mmol/l. Stock solutions of Bz-DL-Arg-OpNAn (2 mmol/l) were diluted with Tris-containing Bz-D-Arg-OpNAn (1 mmol/l) and Tris to obtain a constant concentration of Bz-D-Arg-OpNAn (0.25 mmol/l). The plasmin

TABLE I

Apparent Michaelis constants (K_m) and maximal velocities (V) of the plasmin-catalysed hydrolysis of Bz-L-Arg-OpNAn at different concentrations of Bz-D-Arg-OpNAn in the reaction mixtures (rm), $y \pm s_y$. For symbols, see Eqns 2 and 4. The values of V refer to the plasmin concentration of the stock solution.

Concn Bz-D-Arg-OpNAn (rm) (mmol·l^{-1})	$K_{m(i)}$ (μmol·l^{-1})	K_m (μmol·l^{-1})	V (μmol·l^{-1}·s^{-1})
0.8	62.7 ± 1.7	60 ± 1.6	1.13 ± 0.042
1.6	67.0 ± 2.3	60 ± 2.0	1.12 ± 0.024
2.4	68.7 ± 2.2	60 ± 1.9	1.13 ± 0.025

TABLE II

The time constant ($-F$) of the slow exponential term and the "burst" size (π) of the presteady-state, plasmin-catalysed hydrolysis of Bz-L-Arg-OpNAn at different concentrations of Bz-L-Arg-OpNAn in the reaction mixture (rm), $y \pm s_y$. For symbols, see Theory. π data are equivalent to the concentration of plasmin in the stock solution.

Concn Bz-L-Arg-OpNAn (rm) (μmol·l^{-1})	$-F$ (s^{-1})	π (μmol·l^{-1})
250	0.400 ± 0.007	4.87 ± 0.05
200	0.342 ± 0.006	4.46 ± 0.05
125	0.245 ± 0.004	3.42 ± 0.05
50	0.150 ± 0.002	1.43 ± 0.05

solution was thawed and diluted with Tris. The mass concentration of plasmin was 40 mg/l in the reaction mixtures.

The linear regression representing the extrapolated steady-state rate, (Eqn 6), and the constant, F (Eqn 8), were calculated for each concentration of substrate as described above. The presteady-state data obtained are shown in Table II.

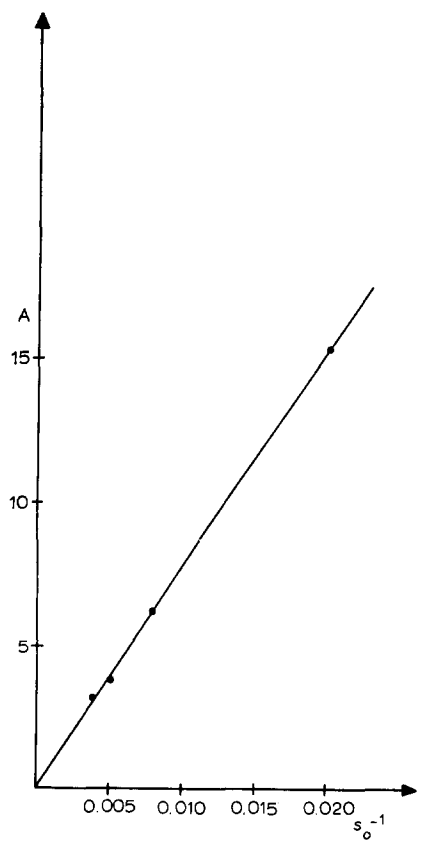

Fig. 2. The regression of $-(1 + K_m/s_0)/F$ on s_0^{-1}. Ordinate (A), $(-(1 + K_m/s_0)/F)$/s; abscissa (s_0^{-1}), the reciprocal of the concentration of Bz-L-Arg-OpNAn in the reaction mixture/l·μmol^{-1}. For symbols, see Theory.

The regression of $-(1 + K_m/s_0)/F$ on $1/s_0$ has $1/k_2$ as intercept and K_s/k_{cat} as slope (Eqn 8). A graph of the regression is shown in Fig. 2. The curve was linear as predicted by the theory, thus supporting the concept of the proposed reaction mechanism (Eqn 1) as a model for the plasmin-catalysed reaction. The kinetic constants determined from steady-state and presteady-state kinetics (see Theory) are shown in Table III.

TABLE III

Kinetic constants of the plasmin-catalysed hydrolysis of Bz-L-Arg-OpNAn in terms of a three-step mechanism with an acyl–enzyme intermediate (Eqn 1), $y \pm s_y$.

Kinetic constant	Value
k_1	$>10^5$ l·mol^{-1}·s^{-1}
k_{-1}	$>10^3$ s^{-1}
k_2	16 ± 6 s^{-1}
k_3	0.09 ± 0.01 s^{-1}
$k_{cat} = k_2k_3/(k_2 + k_3)$	0.086 ± 0.014 s^{-1}
$K_s = (k_{-1} + k_2)/k_1 \approx k_{-1}/k_1$	12 ± 5 mmol·l^{-1}
$K_sk_3/(k_2 + k_3)$	60 ± 2 μmol·l^{-1}

The fast (small) exponential (see Theory) was not detectable in the stopped-flow experiments, and the induction period, τ (Eqn 12) was probably $< 10^{-3}$ s. With $K_s = 12.1$ mmol·l^{-1} and $s_0 \approx 10^{-4}$ mol·l^{-1}, and $k_2 = 15.7\ s^{-1}$ minimal values for the constants k_1 and k_{-1} were approximated in accordance with Eqns 13 and 14 (Table III).

As $k_2 \ll k_{-1}$, K_s approximates the dissociation constant of the enzyme–substrate complex (Eqn 15).

The concentration of active sites of plasmin (e) was calculated by two different methods: (1) By presteady-state kinetics as described in Eqn 11. (2) By titration with the strong polyvalent protease inhibitor. The values obtained are shown in Table IV. The value of e as determined from the presteady-state data, showed no variation with the substrate concentration, supporting the theory of the reaction mechanism (Eqn 1). The values determined by titration by strong inhibitor and the values obtained by the kinetic method were in good agreement.

TABLE IV

THE CONCENTRATION OF ACTIVE SITES OF PLASMIN IN THE STOCK SOLUTION AS ESTIMATED BY TWO METHODS, y $\pm$ *sy*

For symbols, see Theory.

Method	Concn Bz-L-Arg-OpNAn (rm) (μmol·l^{-1})	e (μmol·l^{-1})
Bz-L-Arg-OpNAn hydrolysis		
$e = \pi\ (1 + K_m/s_0)^2$	250	7.4 ± 0.2
	200	7.6 ± 0.2
	125	7.5 ± 0.2
	50	7.0 ± 0.2
Titration with polyvalent protease inhibitor		8.5 ± 0.6

TABLE V

KINETIC CONSTANTS FOR THE HYDROLYSIS OF DIFFERENT SUBSTRATES AS CATALYSED BY PLASMIN AND TRYPSIN

Substrate	Enzyme	k_2/K_s ($l \cdot mol^{-1} \cdot s^{-1}$)	k_2 (s^{-1})	k_3 (s^{-1})	K_s ($mol \cdot l^{-1}$)	K_m ($mol \cdot l^{-1}$)	k_{cat} (s^{-1})
p-Nitrophenyl-*p'*-guanidinobenzoate [2]	plasmin	$4.07 \cdot 10^4$	0.46	$5.2 \cdot 10^{-6}$	$1.13 \cdot 10^{-5}$	$1.3 \cdot 10^{-10}$	$5.2 \cdot 10^{-6}$
p-Nitrophenyl-*p'*-guanidinobenzoate [3]	plasmin	$1.185 \cdot 10^4$	$6.4 \cdot 10^{-2}$	0	$5.2 \cdot 10^{-6}$	0	0
N-(*p*-Carboxybenzyl) pyridinium-*p*-nitrophenyl ester [3]	plasmin	$1.507 \cdot 10^2$	$8.4 \cdot 10^{-2}$	0	$5.3 \cdot 10^{-4}$	0	0
α-*N*-Benzoyl-L-arginine-*p*-nitroanilide	plasmin	$1.34 \cdot 10^3$	16	$9 \cdot 10^{-2}$	$1.2 \cdot 10^{-2}$	$6 \cdot 10^{-5}$	$9 \cdot 10^{-2}$
p-Nitrophenyl-*p'*-guanidinobenzoate [3]	trypsin	$3.175 \cdot 10^6$	1.95	$3.4 \cdot 10^{-5}$	$6.1 \cdot 10^{-7}$	$1.1 \cdot 10^{-11}$	$3.4 \cdot 10^{-5}$
N-Benzoyl-L-arginine-*p*-nitroanilide [11]	trypsin	$1.5 \cdot 10^3$	—	—	—	$9 \cdot 10^{-4}$	0.611
p-Nitrophenyl-α-*N*-benzyloxocarbonyl-L-lysine [4]	trypsin	$1.09 \cdot 10^3$	0.395	$1.43 \cdot 10^{-2}$	$3.5 \cdot 10^{-4}$	$1.27 \cdot 10^{-5}$	$1.4 \cdot 10^{-2}$
α-*N*-Methyl-α-*N*-(*p*-toluenesulfonyl)-L-lysine-β-naphthyl ester [5]	trypsin	$6.56 \cdot 10^3$	5.7	$5.83 \cdot 10^{-2}$	$8.8 \cdot 10^{-4}$	$8.8 \cdot 10^{-6}$	$5.8 \cdot 10^{-2}$

DISCUSSION

The serine proteases constitute an important group of enzymes in animal physiology. Plasmin is a serine protease originating from an inactive precursor, plasminogen, which is present in animal blood plasma. Plasmin catalyses the splitting of α-*N*-L-lysine- and α-*N*-L-arginine-substituted esters [14, 15], amides (present paper), and peptide bonds involving the same amino acids in a number of different proteins. Although there are many similarities between the catalytic properties and specificity of trypsin and plasmin there are also distinct differences with regard to high molecular substrates [16, 17].

Plasmin was found to possess a sequence of active site amino acids, including the serine with the reactive hydroxyl group, which is very similar to that of trypsin, thrombin and chymotrypsin [18]. This suggests that a common main mechanism exists for the bond-breaking catalysis and that other residues are responsible for the varying specificities.

In order to solve the problem of reaction mechanism, many laboratories have done much work investigating reactions catalysed by trypsin and chymotrypsin using a variety of approaches [1]. It is generally assumed that the mechanism involves the formation of an intermediate by acylation of the reactive serine residue of the enzyme (Eqn 1).

The steady-state and presteady-state kinetic data obtained in this study are satisfactorily described by the three-step mechanism of Eqn 1. The results are then consistent with the assumption of a mechanism where Bz-L-Arg-OpNAn is hydrolysed by plasmin in a single-pathway reaction with a α-*N*-benzoyl-L-arginine–acyl–plasmin complex as intermediate. This reaction mechanism has previously been shown to account for the hydrolysis of *p*-nitrophenyl-*p'*-guanidinobenzoate [2, 3] and *N*-(*p*-carboxybenzyl) pyridinium-*p*-nitrophenyl ester [3] by plasmin. Some values of kinetic constants are shown in Table V. Comparing the values for k_2/K_s ($= k_{cat}/K_m$) which is significant in the consideration of correlations between substrate structure and enzyme specificity [1], it is seen that the value for the plasmin reaction with Bz-L-Arg-OpNAn is of the same order of magnitude as the values for the trypsin reactions with substrates usually regarded as specific substrates, whereas the values for both the plasmin and the trypsin reaction with less specific substrates as *p*-nitrophenyl-*p'*-guanidinobenzoate and *N*-(*p*-carboxybenzyl) pyridinium-*p*-nitrophenyl ester are very different. It may thus be allowed to regard Bz-L-Arg-OpNAn as a specific plasmin substrate and to conclude that the normal plasmin reaction pathway is satisfactorily described by Eqn 1, similar to that of trypsin and chymotrypsin.

Specific titration procedures have definite advantages over rate assays for the determination of the concentration of enzymes. The former employs as a standard a solution of a stable low molecular compound at a known concentration. The latter employs as a standard a solution of a labile enzyme preparation and the procedure is complicated by the large number of variables involved in rate assays.

The present work contains a comparison of two different methods of determining the concentration of the active sites of plasmin. One method is based on the "burst" production of a coloured product of the reaction of plasmin with Bz-L-Arg-OpNAn resulting in the formation of an acyl–enzyme intermediate (Eqn 1). The concentration of the product liberated is equivalent to the active site concentration of the

enzyme and may yield a good approach to the true value of the active site concentration.

The other method employed is based on the strong (probably irreversible) binding of plasmin to the polyvalent protease inhibitor, identical to the inhibitor first isolated by Kunitz and Northrop [19] from bovine pancreas. The point of equivalence is indicated by the complete inhibition of enzymatic activity against a suitable plasmin substrate, in the present case, Bz-L-Arg-OEt. This titration is simple to perform but may give erroneous results because of unspecific binding of the inhibitor to plasminogen, denatured plasmin or other contaminating proteins. In the present work the two methods of active site titrations yielded comparable results within the error of the methods.

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