Catalytic Properties of Human Urinary Kallikrein. 1†

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ABSTRACT: Kinetic studies have been carried out with a well-characterized preparation of human urinary (h.u.) kallikrein using chromogenic substrates. Steady-state and pre-steady-state data for h.u. kallikrein catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester (ZLysONp) and of N^{α}-carbobenzoxy-L-alanine *p*-nitrophenyl ester (ZAlaONp) in the presence and absence of ethylamine and acetamidine have been obtained under various conditions and have been analyzed in the framework of the minimum three-step mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}P + P_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P_2$$

The pH dependencies of the kinetic parameters for the hydrolysis of ZLysONp and of ZAlaONp in the presence of saturating levels of ethylamine and acetamidine show that at acid pH values (≤4) the k_3 step (deacylation) is rate limiting in catalysis, whereas for pH values ≥6, k_2 (acylation) becomes rate limiting. On the other hand, the acylation step is rate limiting in the enzymatic hydrolysis of ZAlaONp over the whole pH range explored. Saturating concentrations of acetamidine increase, more than those of ethylamine, k_{cat} for the hydrolysis of ZAlaONp. The affinity of h.u. kallikrein for acetamidine and ethylamine changes about 5-fold with pH between pH 5 and 3. The pH dependence of the spectral properties of free h.u. kallikrein reflects the ionization of a group with a pK_a value of 4.45 ± 0.1. The results point out that, similarly to bovine β-trypsin, h.u. kallikrein catalysis involves an ionizable group which has a pK_a of about 4.5 in the free enzyme and a pK_a of about 3.7 in the enzyme bound to cationic substrates or ligands.

Human urinary (h.u.)[1] kallikrein belongs to the glandular kallikreins. This enzyme is a serine protease, which releases the pharmacologically active kinins from kininogens of the blood plasma (Carretero & Scili, 1978; Mills, 1979; Overlack et al., 1979).

The functions of glandular kallikreins appear to be closely related to the function of the organs in which the enzymes are found. In this respect the excretion of kallikrein in the urine reflects the activity of renal kallikrein, which has been suggested to regulate renal blood flow in cooperation with the renin–angiotensin system in kidney (Mills et al., 1976; Horwitz et al. 1978; Pisano et al., 1978). The work reported here aims to a better definition of the catalytic properties of h.u. kallikrein.

Generally, the study of the catalytic properties of proteolytic enzymes is appreciably simplified by the use of chromogenic substrates showing favorable kinetic parameters. Unfortunately, the steady-state parameters for the h.u. kallikrein catalyzed hydrolysis of anilides, which may be directly followed spectrophotometrically, at 405 nm, are unfavorable (Claeson et al., 1978). Because of this, TosArgOMe and BzArgOEt are commonly employed in the study of the catalytic properties of h.u. kallikrein (Hial et al., 1974; Matsuda et al., 1976; Oza & Ryan, 1978; Ole-MoiYoi et al., 1979), although with these substrates the hydrolysis can be directly followed only in the far-ultraviolet region.

It has been shown before that ZLysONp is a favorable substrate for both serine (Silverstein, 1957; Bender et al., 1966; Ascenzi et al., 1980; Ascenzi et al., 1981; Antonini & Ascenzi, 1981) and thiol (Bender & Brubacher, 1966; Fink & Bender, 1969; Hollaway & Hardman, 1973) proteases, and it would appear to have some of the ideal properties as a chromogenic substrate for h.u. kallikrein, particularly in studies on h.u. kallikrein inhibitors which are in progress in our laboratories. The potentially wide utilization of ZLysONp as a substrate stimulated interest in a detailed study of the pre-steady-state and steady-state kinetics of the h.u. kallikrein catalyzed hydrolysis of ZLysONp between pH 3.5 and 8.5.

The effect of acetamidine and ethylamine, which may be considered as the side chains of lysine and arginine, on the kinetic parameters of h.u. kallikrein catalyzed hydrolysis of ZAlaONp has been investigated. Spectral properties of free and cation-bound h.u. kallikrein are also described.

In the following paper (Ascenzi et al., 1982), a comparison of the catalytic properties of h.u. kallikrein with those of several serine proteases will be reported.

Materials and Methods

Human urinary kallikrein, kindly provided by "Lepetit S.p.A." was prepared according to Geiger et al. (1980). The molecular weights of the native and neuraminidase-treated enzyme (see below) were measured by (i) polyacrylamide gel electrophoresis in 1% $NaDodSO_4$ in the presence and absence of 1% mercaptoethanol [according to Weber et al. (1972)], (ii) gel filtration on Ultrogel AcA44 (LKB) and on Sephadex G-200 [according to Amouric & Figarella (1980)], (iii) titration of the active site of the enzyme with *p*-nitrophenyl *p*-guanidinobenzoate [according to Fiedler et al. (1972)], and (iv) sedimentation velocity experiments using a Beckman Spinco E analytical ultracentrifuge.

The N-terminal residue was determined for both the native and neuraminidase-treated molecule as previously reported (Woos & Wang, 1967; Hartley, 1970). Isoelectric focusing

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[1] Abbreviations: h.u. kallikrein, human urinary kallikrein; ZLysONp, N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester; ZAlaONp, N^{α}-carbobenzoxy-L-alanine *p*-nitrophenyl ester; TosArgOMe, N^{α}-tosyl-L-arginine methyl ester; BzArgOEt, N^{α}-benzoyl-L-arginine ethyl ester; ZAla, N^{α}-carbobenzoxy-L-alanine; ZLys, N^{α}-carbobenzoxy-L-lysine; $NaDodSO_4$, sodium dodecyl sulfate.

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experiments (pH gradient from 3 to 5) were performed according to Vesterberg & Svenssorn (1966) by using an LKB apparatus. The biological activity of h.u. kallikrein was determined as described by Frey et al. (1968) by using the increase of arterial blood flow in dogs and expressed in KE units (Frey et al., 1968).

Neuraminidase-treated h.u. kallikrein was prepared according to Fritz et al. (1967). Neuraminidase, from *Vibrio cholerae*, was obtained from Calbiochem-Behring Co. The presence (in the native molecule) or absence (in the treated molecule) of sialic acid was checked by the Resorcin test (Warren, 1959; Whitehouse & Zilliken, 1960).

The concentration of h.u. kallikrein was determined by using the following extinction coefficients at 280 nm (pH 6.80, phosphate buffer $I = 0.1$ M, $T = 20$ °C); $E^{1\%}_{1cm} = 15.9$ and $E^{1\%}_{1cm} = 16.9$ for the native and neuraminidase-treated species, respectively.

In all the reported experiments, native h.u. kallikrein has been employed. Controls with neuraminidase-treated h.u. kallikrein showed no differences in kinetic, spectral, and binding properties.

ZLysONp and ZAlaONp were obtained from Sigma Chemical Co. *p*-Nitrophenyl *p*-guanidinobenzoate was obtained from Serva Feinbiochemia. Ethylamine and *p*-nitrophenol were obtained from Merck. Acetamidine was obtained from Ega-Chemie. The ethylamide derivatives of ZLys and ZAla were synthesized according to Jones et al. (1973).

The pH dependence of the spectral properties of ZLysONp and ZAlaONp and of the products of the hydrolysis has been previously reported (Ascenzi et al., 1980; Antonini & Ascenzi, 1981; P. Ascenzi, G. Sleiter, and E. Antonini, unpublished results). The second-order rate constants for the alkaline hydrolysis of ZLysONp and ZAlaONp show values, at 20.5 °C, of 1×10^4 M^{-1} s^{-1} and 1.26×10^2 M^{-1} s^{-1}, respectively (Ascenzi et al., 1980; Antonini & Ascenzi, 1981; P. Ascenzi, G. Sleiter, and E. Antonini, unpublished results). Under all the experimental conditions, the initial velocities were corrected for the alkaline hydrolysis of substrates.

Calcium was never present in the reaction mixtures; control experiments showed that the addition of calcium (chloride), up to 1×10^{-2} M, did not modify the catalytic properties of h.u. kallikrein.

Ascending thin-layer chromatography of substrates and products of h.u. kallikrein catalyzed hydrolysis of ZLysONp and ZAlaONp, in the absence and presence of ethylamine and acetamidine, was performed on precoated TLC plates of silica gel F 254 (from Merck) by using the following solvent systems (Tomatis et al., 1978): (1) butan-1-ol–acetic acid–water (15:5:5 v/v); (2) ethyl acetate–pyridine–acetic acid–water (60:20:6:11 v/v); (3) chloroform–methanol–benzene (85:10:5 v/v). The ZAla and ZLys derivatives were located by spraying the chromatograms with a modified chloride reagent (Barrolier, 1961). ZAla, ZLys, *p*-nitrophenol, and amides of both the N^{α}-carbobenzoxy amino acids have been used as markers.

As in the case of bovine β-trypsin (Antonini & Ascenzi, 1981), under all the experimental conditions, the products of the enzymatic hydrolysis of ZLysONp and ZAlaONp, in the presence and absence of ethylamine, are the corresponding N^{α}-carbobenzoxy amino acids and *p*-nitrophenol. The amide derivatives of the N^{α}-carbobenzoxy amino acids have never been observed also when the analysis was done during the progress of the hydrolysis process.

The pH profile was investigated by using the following buffers [all at 0.1 M (sodium salts)]: phosphate, pH 2–3.5; acetate, pH 3.5–6; phosphate, pH 6–8.5. Control experiments with overlapping buffers showed no specific anion effects.

The spectrophotometric measurements were carried out with a double beam spectrophometer (Cary 118 or 219) or with a Gibson-Durrum stopped-flow apparatus equipped with a 2-cm observation chamber. All the measurements were performed at 21 ± 1 °C.

Determination of the Equilibrium and Kinetic Constants. The steady-state and pre-steady-state data for the enzyme-catalyzed hydrolysis of ZLysONp and ZAlaONp were analyzed (Gutfreund & Sturtevant, 1956; Gutfreund, 1972; Hollaway et al., 1971) in the framework of the minimum three-step mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}P + P_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P_2 \qquad (1)$$

where P_1 is *p*-nitrophenol and P_2 the N^{α}-carbobenzoxy L-amino acid.

Values of k_{cat} and K_m have been determined from the intercepts on the ordinate and abscissa, respectively, of a plot of 1/initial rate vs. $1/[S_0]$ with $[S_0] \gg [E_0]$. Values of k_2 and K_s have been determined from the intercepts on the ordinate and abscissa, respectively, of a plot of $1/k_{app}$ vs. $1/[E_0]$ with $[E_0] \gg [S_0]$. The value of k_3 was calculated by substitution of the values of k_2, K_s, k_{cat}, and K_m into

$$k_3 = -\frac{k_{cat}k_2}{k_{cat} - k_2} \qquad (2)$$

$$k_3 = -\frac{K_m k_2}{K_m - K_s} \qquad (3)$$

The values of k_3, calculated from eq 2 and 3, were the same within the errors.

When $[S_0] \geq [E_0]$, the differential equations arising from mechanism 1 may be solved to describe the time course of *p*-nitrophenol release (P_1) (Gutfreund, 1972):

$$P_1 = \frac{k_{cat}[E_0]t}{\left\{1 + \frac{K_m}{[S_0]}\right\}} + \alpha_1[E_0](1 - e^{-kt}) \qquad (4)$$

According to eq 4, when $k_2 \gg k_3$, a "burst" of *p*-nitrophenol release of amplitude A ($=\alpha_1[E_0]$, i.e., $\alpha_2[S_0]$), with a first-order rate constant k, is experimentally detectable.

An average error value of ±8% was evaluated for k_2, K_s, k_{cat}, and K_m according to Atkins & Nimmo (1973).

The values of the dissociation equilibrium constants for the binding of acetamidine and ethylamine to h.u. kallikrein have been obtained (i) by the promoter effect on the h.u. kallikrein catalyzed hydrolysis of ZAlaONp [according to Inagami & Mitsuda (1964); Inagami & Murachi, 1964; Antonini & Ascenzi, 1981], (ii) by the inhibitory effect on the h.u. kallikrein catalyzed hydrolysis of ZLysONp [according to Dixon & Webb (1964)], and (iii) by spectrophotometric titrations.

In the cation binding experiments, the (enzyme·ligand) *minus* (enzyme and ligand) spectra were recorded from 230 to 260 nm as previously reported (Benmouyal & Trowbridge, 1966; East & Trowbridge, 1968). Tandem cells (Hellma, catalog no. 238) were employed so that the reactants were mixed in the sample beam but kept separate in the reference beam. Single and serial additions of stock cationic solutions were made with an AGLA microsyringe directly into the appropriate cell compartments. The titrations conform to a simple equilibrium with a value of the Hill coefficient $n = 1$ (Wyman, 1964).

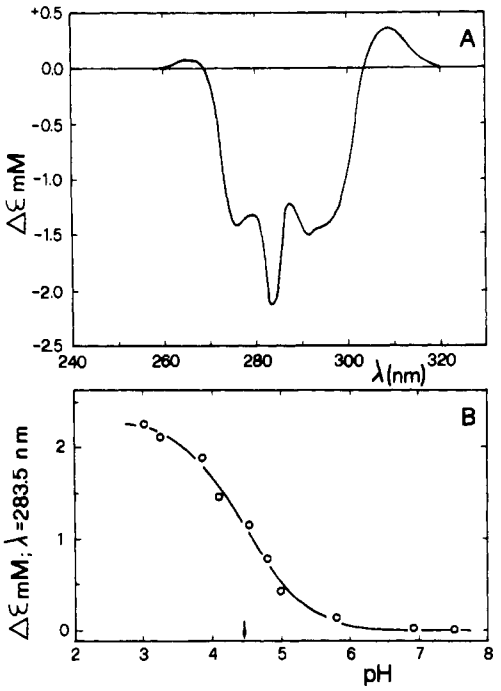

FIGURE 1: (A) pH difference spectrum of h.u. kallikrein. The reference solution was at pH 7.5, and the sample solution was at pH 2.8 (phosphate buffers all at 0.1 M ionic strength, $T = 21 \pm 1$ °C). (B) Spectrophotometric pH titration of h.u. kallikrein. The solid line is the theoretical curve for one ionizable group with a pK_a value of 4.45. The data have been obtained as reported under Materials and Methods ($T = 21 \pm 1$ °C).

The enzyme pH difference spectra were obtained [according to East & Trowbridge (1968)] with 1-cm cells; the reference solution was held at about pH 7.5, while serial additions of 0.1 M phosphoric or acetic acid were added to the sample solution. In parallel the same volume of distilled water was added in the reference cell.

Results

Characterization of Human Urinary Kallikrein. Analysis of the N-terminal amino acid residue of native and neuraminidase-treated h.u. kallikrein showed that the N-terminal was isoleucine, for both proteins, in agreement with previous data (Lottspeich et al., 1979; Geiger et al., 1980).

Polyacrylamide gel electrophoresis in 1% $NaDodSO_4$ in the presence and absence of 1% mercaptoethanol of native h.u. kallikrein showed a single band of apparent molecular weight 31 000 ± 500. This value is in agreement with those obtained by gel filtration on Ultrogel AcA44 (LKB) (31 000 ± 500) and on Sephadex G-200 (30 700 ± 500) and by the titration of the active site of the enzyme with *p*-nitrophenyl *p*-guanidinobenzoate (30 800 ± 500). These values may be regarded as higher limits.

The native and neuraminidase-treated enzymes (pH 7, phosphate buffer 0.1 M, $T = 20$ °C) show sedimentation coefficients of 2.8 and 2.6 S, respectively, at a protein concentration of 3 mg/mL.

The molecular weight of the enzyme is affected by treatment with neuraminidase. The molecular weight of the treated molecule, obtained with different techniques, corresponded to 25 000 ± 500. This value is in agreement with the molecular weight value of the protein moiety of the enzyme deduced from the amino acid composition and from preliminary data on the primary structure (Lottspeich et al., 1979; Geiger et al., 1980). The change in molecular weight after treatment with neuraminidase reflects the loss of the sialic acid residues of the glycoprotein enzyme. It is relevant to observe that in porcine pancreatic kallikrein, sialic acid contributes only 1% of the molecular weight of the enzyme (Fritz et al., 1967).

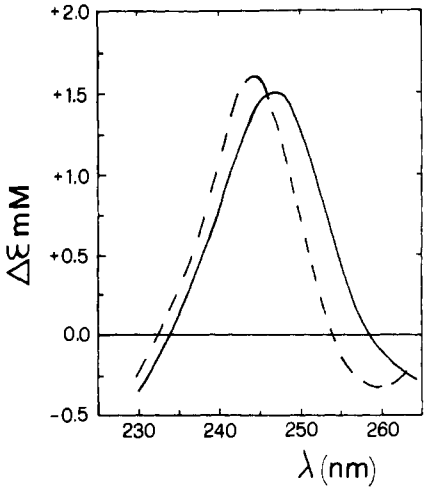

FIGURE 2: Difference spectra of ethylamine (—) and acetamidine (- - -) h.u. kallikrein complexes, against the free enzyme at pH 7.5 (0.1 M phosphate buffer, $T = 21 \pm 1$ °C; 0.2 M ethylamine and acetamidine).

Isoelectric focusing experiments show the presence of only one component with a p*I* value of 3.95. Treatment with neuraminidase induces a change in the p*I* value to 4.05.

At low pH (pH ≤3) h.u. kallikrein is inactive; however, the enzyme brought back to neutral pH shows complete recovery of the activity.

The absorption spectra of free h.u. kallikrein and of its complexes with acetamidine and ethylamine have been investigated as a function of pH. Figure 1A shows the pH difference spectrum of h.u. kallikrein between 230 and 330 nm. The pH dependence of the spectral properties of the free enzyme reflects the ionization of a group with a pK_a value of 4.45 ± 0.1 (Figure 1B). No pH difference spectral changes of free h.u. kallikrein are present between 230 and 260 nm; over this wavelength range, absorption changes accompanying ethylamine and acetamidine binding have been observed (Figure 2). The difference spectra, between 230 and 260 nm, of h.u. kallikrein in the presence of saturating concentrations of ethylamine and acetamidine, against the free enzyme, are pH independent.

Steady-State Parameters of Human Urinary Kallikrein Catalyzed Hydrolysis of ZLysONp. When catalytic concentrations of h.u. kallikrein are used, ZLysONp can be usefully employed as a substrate only up to pH 8.2, since spontaneous hydrolysis becomes considerable at pH values above 7 (Ascenzi et al., 1980; P. Ascenzi, G. Sleiter, and E. Antonini, unpublished results).

The dependence of initial velocity on the concentration of substrate and the time course of the reaction conform to simple Michaelis–Menten kinetics (Figure 3A). Over the wide range explored (5×10^{-9}–5×10^{-7} M), the initial velocity is strictly linear with enzyme concentration (Figure 3B).

Figure 4 shows the pH dependence of log k_{cat}/K_m for the h.u. kallikrein catalyzed hydrolysis of ZLysONp. The data can be fitted with two pH transitions having pK_a values of 4.50 ± 0.1 and 7.00 ± 0.1. The value of K_m is pH independent, within experimental error, with an average value of 90 ± 10 μM. The limiting value of k_{cat} is 54 s^{-1}, one of the fastest known in h.u. kallikrein catalysis.

Effect of Acetamidine and Ethylamine on Steady-State Parameters of Human Urinary Kallikrein Catalyzed Hydrolysis of ZAlaONp. It is known that the rate of the trypsin-catalyzed hydrolysis of neutral substrates is increased in

Table I: Values of Steady-State Parameters and Individual Rate Constants for Human Urinary Kallikrein Catalyzed Hydrolysis of ZLysONp and ZAlaONp in the Presence and Absence of Saturating Levels of Ethylamine and Acetamidine (T = 21 ± 1 °C)

substrate	pH	k_2 (s^{-1})	K_s (μM)	k_2/K_s (μM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	k_3 (s^{-1})	$\alpha_2 = A/[S_0]^a$	k^a (s^{-1})
ZLysONp	3.90	3.0	700	0.0043	0.4	100	0.0040	0.5	0.12	1.3
	6.20	10.0	120	0.083	7	91	0.077	30.0		
ZAlaONp	3.95	0.005	100	5×10^{-5}	0.003	75	4×10^{-5}	0.01		
	5.90	0.15	130	1.2×10^{-3}	0.1	100	1×10^{-3}	0.4		
ZAlaONp (+ethylamine, 0.2 M)	4.05	0.015	140	1.1×10^{-4}	0.007	60	1.2×10^{-4}	0.01	0.10	0.03
	5.85	0.3	135	2.2×10^{-3}	0.25	120	2.1×10^{-3}	1.8		
ZAlaONp (+acetamidine, 0.1 M)	4.00	0.05	160	3.1×10^{-4}	0.02	70	2.9×10^{-4}	0.035	0.12	0.11
	5.90	1.0	150	6.7×10^{-3}	0.7	130	5.4×10^{-3}	4.5		

[a] $[S_0]$ = 250 μM; $[E_0]$ = 60 μM.

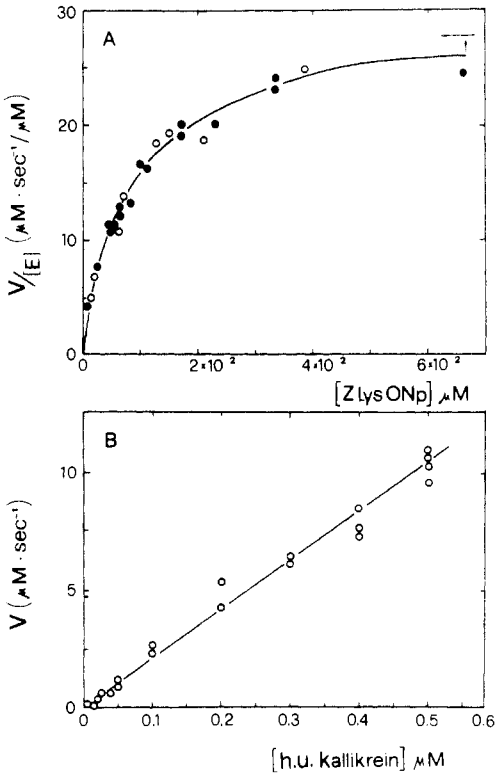

FIGURE 3: (A) Dependence on substrate concentration of the initial velocity for the h.u. kallikrein catalyzed hydrolysis of ZLysONp. Full symbols (●) indicate values of initial velocity measured at a constant concentration of h.u. kallikrein (5×10^{-8} M) and variable concentration of substrate. Open symbols (○) indicate the values obtained from a rate analysis of a single time-course hydrolysis at an initial concentration of ZLysONp of 4.6×10^{-4} M and at a concentration of the enzyme of 5×10^{-8} M (pH 6.8, 0.1 M phosphate buffer, T = 21 ± 1 °C). (B) Dependence of the initial velocity for the hydrolysis of ZLysONp on the concentration of h.u. kallikrein at a constant concentration of substrate (2.28×10^{-4} M). For experimental conditions, see part A.

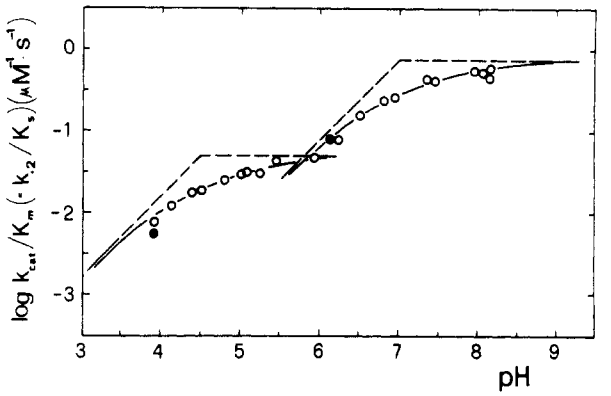

FIGURE 4: pH dependence of log k_{cat}/K_m for the h.u. kallikrein catalyzed hydrolysis of ZLysONp (○). Filled circles (●) indicate values of log k_2/K_s calculated from pre-steady-state measurements. The solid line is the theoretical curve for two ionizable groups with pK_a values of 4.50 and 7.00. The data have been obtained in phosphate buffer, pH 2–3.5, acetate buffer, pH 3.5–6, and phosphate buffer, pH 6–8.5, all 0.1 M; T = 21 ± 1 °C.

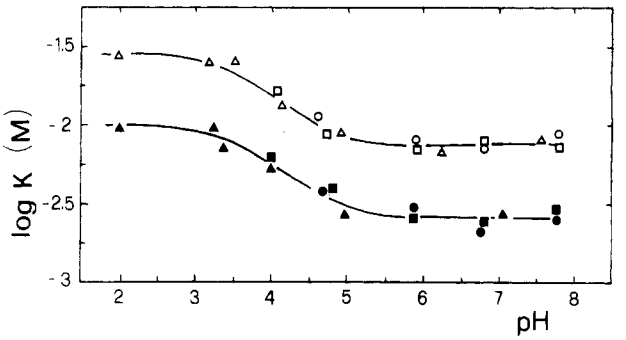

FIGURE 5: pH dependencies of the dissociation constant for the reaction of ethylamine (open symbols) and acetamidine (filled symbols) with h.u. kallikrein. The data have been obtained (i) by the promoter effect of cations on the enzymatic hydrolysis of ZAlaONp (circles), (ii) by the inhibition effect on the hydrolysis of ZLysONp (squares), and (iii) by spectrophotometric titrations (triangles). The solid lines are the theoretical curves for one ionizable group with a pK_a value of 4.10. For experimental conditions, see Figure 4.

the presence of alkylamino compounds (Inagami & Mitsuda, 1964; Inagami & Murachi, 1964; Antonini & Ascenzi, 1981). In view of this, the effect of ethylamine and acetamidine on the hydrolysis of the neutral substrate ZAlaONp by h.u. kallikrein was investigated.

The dependence of the initial velocity on the concentration of ZAlaONp, in the presence or absence of ethylamine or acetamidine, follows simple Michaelis–Menten kinetics.

The kinetic parameters for the h.u. kallikrein catalyzed hydrolysis of ZAlaONp, in the presence or absence of ethylamine and acetamidine, are reported in Table I. The presence of saturating concentrations of acetamidine has been found to increase the initial rate of hydrolysis of ZAlaONp, by about 1 order of magnitude over the whole pH range explored (pH 3.5–8). On the other hand a smaller enhancement, in the value of k_{cat}, has been observed in the presence of saturating levels of ethylamine.

The values of the dissociation constants for the reaction of ethylamine and acetamidine with h.u. kallikrein at different pHs are reported in Figure 5. It may be noted that the affinity for acetamidine is higher than that for ethylamine. The pH dependencies of both the dissociation constants may be fitted by a simple pH transition with a pK_a value of 4.10 ± 0.1. The values of the dissociation constants, determined by the different procedures, indicated under Material and Methods, agree well with one another (see Figure 5).

The independence of K_m (85 ± 10 μM) for ZAlaONp hydrolysis on the concentration of acetamidine or ethylamine over

the explored pH range shows that the binding of the positively charged molecules does not affect the binding of ZAlaONp. Therefore the accelerating effect on catalysis of acetamidine and ethylamine may be ascribed exclusively to an enhanced activity of the catalytic site as reflected by the increase of k_{cat} (see Table I).

Pre-Steady-State Kinetics of the Human Urinary Kallikrein Catalyzed Hydrolysis of ZLysONp and of ZAlaONp in the Absence and in the Presence of Saturating Levels of Acetamidine and Ethylamine. The determination of the kinetic parameters of mechanism 1 is simplified by the fact that the process $E + S \rightleftharpoons (k_1, k_{-1})$ E·S (with $K_s = k_{-1}/k_1$) may be regarded as being complete within 3 ms, "dead time" of the stopped-flow apparatus, so that $600\ s^{-1} \leq k_{-1} \gg k_2$. Taking the value of $k_{-1} = 600\ s^{-1}$ from the value of K_s, the minimum values for the rate constant for the formation of the E·S complex are $5 \times 10^6\ M^{-1}\ s^{-1}$ (pH 6.2) and $8.6 \times 10^5\ M^{-1}\ s^{-1}$ (pH 3.9) for ZLysONp and $5.2 \times 10^6\ M^{-1}\ s^{-1}$ for ZAlaONp over the whole pH range explored (pH 4–6); the latter is unaffected by the presence of saturating levels of acetamidine and ethylamine.

Addition of *p*-nitrophenol (up to 50 μM) in the reaction mixtures did not affect the rate of release of *p*-nitrophenol from ZLysONp and ZAlaONp. Thus it may be postulated that $k_{-2} \approx 0$.

The values of K_s, k_2, and k_3 obtained at different pHs for the hydrolysis of ZLysONp and of ZAlaONp in the absence and presence of acetamidine and ethylamine are reported in Table I.

The most significant result shown in Table I is the pH dependence of the rate-limiting step of the enzymatic hydrolysis of ZLysONp and of ZAlaONp in the presence of saturating levels of ethylamine and acetamidine. Thus, at pH ≤ 4, k_3 is limiting in catalysis, whereas at pH ≥ 6, k_2 becomes rate limiting. In parallel with the change in the rate-limiting step, below pH 6, the value of K_s is pH dependent, indicating a change in the pK_a of a group on binding the substrate. On the other hand, the acylation step (k_2) is rate limiting in the hydrolysis of ZAlaONp in the absence of ethylamine and acetamidine, over the whole pH range explored (pH 4–6). Under the conditions where k_3 is rate limiting in catalysis, a burst phase in the release of *p*-nitrophenol (of amplitude $A = \alpha_2[S_0]$ and rate constant k) is detectable (see Table I).

Discussion

The present paper reports a characterization of the catalytic properties of h.u. kallikrein. The h.u. kallikrein preparation used in the present study was proved to be homogeneous by a number of criteria. The molecular weight of the neuraminidase-treated enzyme is about 25 000 and is lower than that of the native enzyme. However, no difference in catalytic, spectral, and cation binding properties has been observed between the whole and the carbohydrate-depleted enzyme.

The catalytic parameters for the hydrolysis of ZLysONp by h.u. kallikrein compare favorably with those reported for other synthetic substrates and indicate that it may be the substrate of choice for h.u. kallikrein as for other serine proteases acting on cationic substrates (Silverstein, 1957; Bender et al., 1966; Ascenzi et al., 1980, 1981; Antonini & Ascenzi, 1981). This is also made clear by the data reported in the following paper (Ascenzi et al., 1982) in which values of k_{cat} and K_m for a number of ester and anilide amino acid derivatives are given. Under the conditions used, k_{cat} is approximately 5-fold greater for ZLysONp as compared to that for TosArgOMe or BzArgOEt which are commonly used for the assay of h.u. kallikrein (Hial et al., 1974; Matsuda et al., 1976; Oza & Ryan, 1978; Ole-MoiYoi et al., 1979).

From the data given above it may be calculated that the assay with ZLysONp, at pH 6.8, allows the determination of h.u. kallikrein at a concentration as low as 5×10^{-9} M corresponding to about 0.3 KE unit/mL. The larger promoting effect of acetamidine than of ethylamine on the enzymatic hydrolysis of ZAlaONp is in agreement with the finding that arginine derivatives are better substrates than the corresponding lysine derivatives (Ascenzi et al., 1982).

The pre-steady-state and steady-state kinetic data for the h.u. kallikrein catalyzed hydrolysis of ZLysONp have been analyzed in detail in the framework of the three-step mechanism (eq 1). The results reported in Table I indicate that the rate-limiting step for the catalyzed hydrolysis of cationic substrates changes with pH, as previously observed for bovine β-trypsin (Ascenzi et al., 1981; Antonini & Ascenzi, 1981). In fact, the rate-limiting step in catalysis is k_3 at pH ≤ 4 and k_2 at pH ≥ 6. The pH dependence of the kinetic parameters for ZLysONp, reported in Table I and Figure 4, implies, in the acidic region, a role for a group with a pK_a of 4.50 ± 0.1 in the free enzyme [according to Peller & Alberty (1959)]. In parallel, the values of K_s increase from pH 6 to 4, reflecting, according to linkage relations (Wyman, 1964), an acid shift in the pK_a value of a group on binding ZLysONp. Similarly, the pH dependence of the dissociation constant for the binding of ethylamine and acetamidine to h.u. kallikrein implies a pK_a shift from 4.50 ± 0.1 in the free enzyme to 3.70 ± 0.1 in the complexes. The absorption spectrum of h.u. kallikrein is pH dependent and may be fitted with a simple titration curve with an average pK_a value of about 4.45 ± 0.1. On the other hand, the absorption spectra of the ligand-bound enzyme are pH independent.

These observations suggest that, similarly to bovine β-trypsin, h.u. kallikrein contains an ionizable residue with a pK of about 4.5 in the free enzyme, identified in trypsin as the carboxylate of Asp-189 (Antonini & Ascenzi, 1981), which plays a critical role in the catalytic mechanism. Recent X-ray data on porcine pancreatic kallikrein (Professor R. Huber and Professor W. Bode, personal communication), which shows large structural similarities with h.u. kallikrein (Lottspeich et al., 1979), indicate that a carboxylic residue at position 189 is present also in this enzyme.

Acknowledgments

We thank Professor R. Huber and Professor W. Bode, who kindly communicated X-ray data about porcine pancreatic kallikrein, Dr. P. Vecchini for performing the ultracentrifuge experiments, and Dr. M. Bolognesi, who read the manuscript and offered constructive criticism. We also thank Professor M. Laskowski, Jr., for stimulating discussions.

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