Catalytic Properties of Serine Proteases. 2. Comparison between Human Urinary Kallikrein and Human Urokinase, Bovine β-Trypsin, Bovine Thrombin, and Bovine α-Chymotrypsin†

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ABSTRACT: The catalytic properties of several serine proteases acting on cationic substrates (bovine β-trypsin, bovine thrombin, human urinary kallikrein, and human urokinase) and noncationic substrates (bovine α-chymotrypsin) have been compared in steady-state and pre-steady-state experiments by using ester and anilide synthetic substrates. Arginine and lysine derivatives are equally good substrates for b. β-trypsin; b. thrombin and h.u. kallikrein prefer substrates containing arginine side chains; h. urokinase prefers substrates containing lysine. The preference of the various enzymes for the guanidinium or ammonium group is reflected by the different promoter effect that acetamidine or ethylamine has on the catalyzed hydrolysis of neutral substrates. Pre-steady-state data, analyzed in the framework of the three-step model, show that for b. β-trypsin, b. thrombin, h.u. kallikrein, and h. urokinase the acylation step (k_2) is rate limiting above pH 6 and the deacylation step (k_3) below pH 4 in the hydrolysis of ZLysONp and of ZAlaONp in the presence of acetamidine or ethylamine. In the catalyzed hydrolysis of ZAlaONp, in the absence of acetamidine or ethylamine, the acylation step (k_2) is rate limiting all over the pH range from 3 to 8. The change in the rate-limiting step with pH is always absent, for the same substrates, in the b. α-chymotrypsin catalysis. The results of kinetic and spectral measurements indicate that b. β-trypsin, b. thrombin, h.u. kallikrein, and h. urokinase, but not b. α-chymotrypsin, contain a similarly located ionizable group with a pK_a of 4.50 ± 0.1, in the free enzyme, the ionization of which affects the binding of cationic substrates and ligands, the spectral properties of the proteases, and the rate of the acylation step in catalysis.

In the previous paper we reported on the catalytic properties of human urinary (h.u.)[1] kallikrein (Antonini et al., 1982). The object of the present paper is to compare side by side the catalytic properties of several serine proteases acting on cationic and noncationic substrates, namely, b. β-trypsin, b. thrombin, h. urokinase, h.u. kallikrein, and b. α-chymotrypsin.

A wealth of information on the chemical structure of these proteins is available (Walsh, 1970; Wilcox, 1970; Blow, 1971; Magnusson, 1971; Birktoft & Blow, 1972; Huber et al., 1974; Bode & Schwager, 1975; Ong et al., 1977; Lottspeich et al., 1979). The three-dimensional structures of b. β-trypsin (Huber et al., 1974; Bode & Schwager, 1975) and b. α-chymotrypsin (Blow, 1971; Birktoft & Blow, 1972) have been solved, and similar information on the other enzymes will probably be soon obtained.

Understanding of structure–function relationships in these enzymes requires equally detailed information on the functional properties. These involve investigation of the catalytic parameters for different substrates and of the equilibria and kinetics of binding of substrates and inhibitors, identification of catalytic intermediates and determination of their rates of formation and break down, and measurements of direct and indirect interactions between binding sites for different ligands.

The aim of this work is to provide such type of information on the several serine proteases mentioned above. The study has been conducted along the following lines of investigation: (1) steady-state and pre-steady-state kinetics of the catalyzed hydrolysis of a number of synthetic substrates having cationic and noncationic side chains (particularly, such studies have been performed with ZLysONp over the widest pH range accessible with this substrate (pH 2.5–8.2); ZLysONp results to be a very favorable substrate for all the proteases studied here), (2) the "promoter" effect of ethylamine and acetamidine on the catalyzed hydrolysis of a "neutral" substrate, ZAlaONp, (3) inhibition by ethylamine, acetamidine, and benzamidine of the catalyzed hydrolysis of ZLysONp, and (4) spectral properties of the enzymes in the free and ligand bound form.

Some of the data for h. urokinase, b. β-trypsin, b. α-chymotrypsin and h.u. kallikrein have been obtained before (Ascenzi et al., 1980, 1981; Antonini & Ascenzi, 1981; Antonini et al., 1982) and are expanded here for the purpose of an homogeneous comparison of the various enzymes. It will be shown that serine proteases acting on cationic substrates show similar features, indicating, among others, a critical functional role of a common carboxylate residue. On the other hand clear differences also emerge among the various enzymes which may be related to subtle variations in specificity.

Materials and Methods

Human urinary kallikrein, kindly provided by Lepetit S.p.A., was prepared according to Geiger et al. (1980). The physicochemical properties of both the native and neuraminidase-

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[1] Abbreviations: h.u. kallikrein, human urinary kallikrein; h. urokinase, human urokinase; b. β-trypsin, bovine β-trypsin; b. α-chymotrypsin, bovine α-chymotrypsin; b. thrombin, bovine thrombin; ZLysONp, N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester; ZAlaONp, N^{α}-carbobenzoxy-L-alanine *p*-nitrophenyl ester; ZTyrONp, N^{α}-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester; BzArgOMe, N^{α}-benzoyl-L-arginine methyl ester; TosArgOMe, N^{α}-tosyl-L-arginine methyl ester; BzArgOEt, N^{α}-benzoyl-L-arginine ethyl ester; TosLysOMe, N^{α}-tosyl-L-lysine methyl ester; BzArgNHNp, N^{α}-benzoyl-L-arginine-*p*-nitroanilide; Bz-DL-LysNHNp, N^{α}-benzoyl-DL-lysine-*p*-nitroanilide; BzLysNHNp, N^{α}-benzoyl-L-lysine-*p*-nitroanilide; AcLysOMe, N^{α}-acetyl-L-lysine methyl ester; AcArgOMe, N^{α}-acetyl-L-arginine methyl ester; BzAlaOMe, N^{α}-benzoyl-L-alanine methyl ester; AcAlaOMe, N^{α}-acetyl-L-alanine methyl ester; TosTyrOBe, N^{α}-tosyl-L-tyrosine benzyl ester; BzTyrOEt, N^{α}-benzoyl-L-tyrosine ethyl ester; AcTyrOEt, N^{α}-acetyl-L-tyrosine ethyl ester; AcTyrNHNp, N^{α}-acetyl-L-tyrosine-*p*-nitroanilide; ZAla, N^{α}-carbobenzoxy-L-alanine; ZLys, N^{α}-carbobenzoxy-L-lysine; $NaDodSO_4$, sodium dodecyl sulfate.

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treated enzymes have been previously reported (Antonini et al., 1982).

Bovine β-trypsin, treated with diphenylcarbamoyl chloride in order to abolish chymotryptic activity, was obtained from Sigma Chemical Co. The b. β-trypsin preparation contained less than 5% of α-trypsin as judged from the kinetics of reaction with *p*-nitrophenyl *p*-guanidinobenzoate (Hruska et al., 1969).

Both forms of h. urokinase [molecular weights 33 000 and 54 000; for details see Ascenzi et al. (1980)] were kindly provided by Serono S.p.A. Under all the experimental conditions the M_r 33 000 and 54 000 species of h. urokinase show no differences in kinetic, spectral, and binding properties.

Bovine α-chymotrypsin was obtained from Worthington Chemical Co. Bovine thrombin was obtained from Sigma Chemical Co.

The concentration of the various enzymes was determined by using the following extinction coefficients, at 280 nm (pH 6.80, phosphate buffer $I = 0.1$ M, $T = 20$ °C): b. β-trypsin, $E^{1\%}_{1cm} = 15.6$ (Quast et al., 1978); b. α-chymotrypsin, $E^{1\%}_{1cm} = 20.4$ (Laskowsky, 1955); b. thrombin, $E^{1\%}_{1cm} = 19.5$ (Winzor & Sheraga, 1964); h. urokinase, $E^{1\%}_{1cm} = 13.2$ and $E^{1\%}_{1cm} = 13.6$ for the species of 33 000 and 54 000 in molecular weight, respectively (White et al., 1966); h.u. kallikrein, $E^{1\%}_{1cm} = 15.9$ and $E^{1\%}_{1cm} = 16.9$ for the native and neuraminidase-treated species, respectively (Antonini et al., 1982).

The homogeneity of the enzymes examined was checked by polyacrylamide gel electrophoresis in 1% $NaDodSO_4$ in the presence and absence of 1% mercaptoethanol [according to Weber et al. (1972)]. The enzyme preparations used contained less than 5% of protein contaminants.

The following compounds were obtained from Sigma Chemical Co.: ZLysONp, ZAlaONp, ZTyrONp, BzArgOMe, TosArgOMe, BzArgOEt, TosLysOMe, BzArgNHNp, AcLysOMe, AcArgOMe, BzAlaOMe, AcAlaOMe, TosTyrOBe, BzTyrOEt, AcTyrOEt, ZAla, and ZLys. Bz-DL-LysNHNp, AcTyrNHNp, and *p*-nitrophenyl *p*-guanidinobenzoate were obtained from Serva Feinbiochemia. Ethylamine, benzamidine, and *p*-nitrophenol were obtained from Merck. Acetamidine was obtained from Ega-Chemie. The ethylamide derivatives of ZAla and ZLys were synthesized according to Jones et al. (1973).

The pH dependence of the spectral properties of ZTyrONp and of the products of the hydrolysis and the second-order rate constant for the alkaline hydrolysis of this molecule (2×10^2 M^{-1} s^{-1}) have been previously reported (Martin et al., 1959; P. Ascenzi, G. Sleiter, and E. Antonini, unpublished results).

The initial velocities for the enzymatic hydrolysis of ZLysONp, ZTyrONp, and ZAlaONp were corrected for the alkaline hydrolysis of substrates (Martin et al., 1959; Antonini & Ascenzi, 1981; P. Ascenzi, G. Sleiter, and E. Antonini, unpublished results). On the other hand, BzArgNHNp, Bz-DL-LysNHNp, AcTyrNHNp, BzArgOEt, TosArgOMe, BzArgOMe, TosLysOMe, AcLysOMe, AcArgOMe, BzAlaOMe, AcAlaOMe, TosTyrOBe, and AcTyrOEt show no appreciable alkaline hydrolysis in the explored pH range.

The hydrolysis of ZLysONp, ZTyrONp, ZAlaONp, BzArgNHNp, Bz-DL-LysNHNp, and AcTyrNHNp was monitored spectrophotometrically between 340 and 420 nm (Martin et al., 1959; Kassel, 1970; Ascenzi et al., 1980; Ascenzi et al., 1981; Antonini & Ascenzi, 1981). The hydrolysis of BzArgOMe, TosArgOMe, BzArgOEt, TosLysOMe, BzAlaOMe, TosTyrOBe, and BzTyrOEt was followed spectrophotometrically between 240 and 275 nm (Hummel, 1959). The hydrolysis of AcLysOMe, AcArgOMe, AcAlaOMe, and AcTyrOEt was measured by the release of ethanol or methanol according to Siegelman et al. (1962).

Steady-state and pre-steady-state parameters were obtained from the experimental data according to the standard treatment [see Antonini et al. (1982)] of the reaction mechanism of serine proteases (Gutfreund & Sturtevant, 1956; Gutfreund, 1972; Hollaway et al., 1971):

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

where K_s ($=k_{-1}/k_1$) is the association constant for the formation of the enzyme substrate complex (E·S), E·P is the acyl intermediate, and P_1 and P_2 are the products of hydrolysis. Kinetic parameters for the enzymatic hydrolysis of BzLysNHNp were obtained from the hydrolysis of Bz-DL-LysNHNp (Mares-Guia & Show, 1965). Calcium was never present in the reaction mixtures; control experiments with added calcium chloride (up to 10^{-2} M) showed no effect of Ca^{2+} on the kinetic constants of the enzymes examined.

Chromatography of substrates and products was performed as previously reported (Antonini & Ascenzi, 1981; Antonini et al., 1982).

Values of the dissociation constant (K_i) for the binding of ethylamine, acetamidine, and benzamidine to the enzymes examined have been evaluated as described before (Antonini et al., 1982). The difference spectra of the enzyme–cationic complexes *minus* the free enzyme and the enzyme pH difference spectra were measured as previously (Antonini et al., 1982). An average error value of ±8% was evaluated for kinetic parameters and K_i according to Atkins & Nimmo (1973).

The following buffers were used [all at 0.1 M (sodium salts)]: phosphate, pH 2–3.5; acetate, pH 3.5–6; phosphate, 6–8.5. No specific ion effects were found by using different buffers overlapping in pH.

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

Results

Hydrolysis of ZLysONp Catalyzed by Bovine β-Trypsin, Bovine α-Chymotrypsin, Bovine Thrombin, Human Urinary Kallikrein, and Human Urokinase. (A) Steady-State Data. ZLysONp hydrolyzes spontaneously at alkaline pH and therefore can be usefully employed as a substrate only up to pH ~8.

For all the enzymes examined, the dependence of the initial velocity on substrate and enzyme concentration and the time course of the reaction follow simple Michaelis–Menten kinetics. The steady-state parameters, k_{cat} and K_m, for the various enzymes are reported in Tables I–IV [for h.u. kallikrein, see Table I of the preceding paper in this issue (Antonini et al., 1982)]. For all the enzymes the values of K_m are essentially independent of pH, within experimental error, between pH 2.5 and 8.5.

Figure 1 shows the dependence on pH of the values of log k_{cat}/K_m. The data for b. β-trypsin, h.u. kallikrein [see Antonini et al. (1982)], h. urokinase, and b. thrombin may be fitted with two pH transitions corresponding to pK_a values of 7.0 ± 0.1 and 4.5 ± 0.1 and those for b. α-chymotrypsin with a single transition of $pK_a = 7.0 \pm 0.1$.

(B) Pre-Steady-State Data. The values of the kinetic parameters within mechanism 1 obtained from pre-steady-state

Table I: Values of Steady-State Parameters and Individual Rate Constants for the Bovine β-Trypsin Catalyzed Hydrolysis of ZLysONp and ZAlaONp in the Presence and Absence of Saturating Levels of Ethylamine and Acetamidine ($T = 21 \pm 1$ °C)

substrate	pH	k_2 (s^{-1})	K_s (μM)	k_2/K_s ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_3 (s^{-1})	$\alpha_2 = A/[S_0]^a$	k^a (s^{-1})
ZLysONp	3.98	15.0	510	0.029	2.0	120	0.018	3.2	0.16	4.0
	5.80	38.0	200	0.19	30.0	180	0.17	200.0		
ZAlaONp	4.00	0.02	120	1.6×10^{-4}	0.015	100	1.5×10^{-4}	0.1		
	5.90	0.69	96	0.007	0.46	72	0.0065	4.2		
ZAlaONp (+ethylamine, 0.2 M)	4.05	0.3	140	0.0021	0.1	50	0.002	0.16	0.08	0.18
	6.05	4.0	120	0.033	3.0	80	0.037	10.0		
ZAlaONp (+acetamidine, 0.2 M)	4.00	0.25	160	0.0016	0.1	60	0.0017	0.18	0.09	0.18
	5.95	3.8	130	0.030	2.6	100	0.026	11.0		

[a] $[S_0] = 250\ \mu M$; $[E_0] = 60\ \mu M$.

Table II: Values of Steady-State Parameters and Individual Rate Constants for the Bovine Thrombin Catalyzed Hydrolysis of ZLysONp and ZAlaONp in the Presence and Absence of Saturating Levels of Ethylamine and Acetamidine ($T = 21 \pm 1$ °C)

substrate	pH	k_2 (s^{-1})	K_s (μM)	k_2/K_s ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}\ \mu M^{-1}$)	k_3 (s^{-1})	$\alpha_2 = A/[S_0]^a$	k^a (s^{-1})
ZLysONp	3.70	3.6	480	7.9×10^{-3}	0.63	100	6.3×10^{-3}	1.27	0.16	2.2
	5.75	11.6	160	5.6×10^{-2}	10.0	135	7.4×10^{-2}	78.0		
ZAlaONp	3.65	0.0012	140	8.6×10^{-6}	0.0008	110	7.3×10^{-6}	0.0060		
	6.02	0.19	150	1.3×10^{-5}	0.15	120	1.3×10^{-3}	0.9		
ZAlaONp (+ethylamine, 0.4 M)	3.78	0.004	180	2.2×10^{-5}	0.002	85	2.3×10^{-5}	0.0032	0.09	0.2
	6.15	0.65	133	4.9×10^{-3}	0.4	100	4×10^{-3}	2.8		
ZAlaONp (+acetamidine, 0.2 M)	3.80	0.02	145	1.3×10^{-4}	0.01	105	9.5×10^{-5}	0.016	0.12	0.3
	6.10	2.0	150	1.3×10^{-2}	1.5	125	1.2×10^{-2}	10.2		

[a] See footnote *a* of Table I.

Table III: Values of Steady-State Parameters and Individual Rate Constants for the Human Urokinase (the M_r 54 000 Species) Catalyzed Hydrolysis of ZLysONp and ZAlaONp in the Presence and Absence of Saturating Levels of Ethylamine and Acetamidine ($T = 21 \pm 1$ °C)

substrate	pH	k_2 (s^{-1})	K_s (μM)	k_2/K_s ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_3 (s^{-1})	$\alpha_2 = A/[S_0]^a$	k^a (s^{-1})
ZLysONp	3.50	0.55	550	0.001	0.15	150	0.001	0.21	0.14	0.4
	5.80	1.3	125	0.0104	1.0	100	0.010	4.75		
ZAlaONp	4.05	0.00033	110	3.0×10^{-6}	0.0002	80	2.5×10^{-6}	0.0007		
	5.83	0.025	115	2.0×10^{-4}	0.015	90	1.7×10^{-4}	0.065		
ZAlaONp (+ethylamine, 0.5 M)	4.05	0.0033	160	2×10^{-5}	0.001	60	1.7×10^{-5}	0.002	0.12	0.016
	5.83	0.14	120	1.2×10^{-3}	0.10	95	1.1×10^{-3}	0.44		
ZAlaONp (+acetamidine, 2.0 M)	4.00	0.0018	150	1.2×10^{-5}	0.0008	60	1.3×10^{-5}	0.0013	0.12	0.003
	5.83	0.055	130	4.2×10^{-4}	0.045	110	4.1×10^{-4}	0.28		

[a] See footnote *a* of Table I.

Table IV: Values of Steady-State Parameters and Individual Rate Constants for the Bovine α-Chymotrypsin Catalyzed Hydrolysis of ZLysONp and ZAlaONp in the Presence and Absence of Saturating Levels of Ethylamine and Acetamidine ($T = 21 \pm 1$ °C)

substrate	pH	k_2 (s^{-1})	K_s (μM)	k_2/K_s ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_3 (s^{-1})
ZLysONp	4.03	0.08	700	1.1×10^{-4}	0.06	640	9.4×10^{-5}	0.6
	5.95	3.80	700	5.4×10^{-3}	2.80	620	4.5×10^{-3}	18.2
ZAlaONp	4.00	0.01	120	8.3×10^{-5}	0.008	100	8×10^{-5}	0.05
	6.00	0.50	125	4.0×10^{-3}	0.30	90	3.3×10^{-3}	1.0
ZAlaONp (+ethylamine, 0.2 M)	4.00	0.005	115	4.3×10^{-5}	0.003	105	3×10^{-5}	0.018
	6.05	0.50	120	4.2×10^{-3}	0.25	85	3×10^{-3}	1.5
ZAlaONp (+acetamidine, 0.2 M)	4.05	0.01	120	8.3×10^{-5}	0.004	115	4×10^{-5}	0.02
	6.10	0.60	125	4.8×10^{-3}	0.25	90	2.8×10^{-3}	1.8

measurements are reported in Tables I–IV [for h.u. kallikrein, see Table I of Antonini et al. (1982)] and, graphically, in Figures 1–3.

Figure 2 reports the pH dependence of k_2 values for the b. β-trypsin and b. thrombin catalyzed hydrolysis of ZLysONp. The k_2 vs. pH profile implicates two ionizing groups with pK_a values of 3.7 ± 0.1 and 7.1 ± 0.1, respectively, in the E·S complex.

The value of K_s for the b. β-trypsin, h. urokinase, h.u. kallikrein, and b. thrombin catalyzed hydrolysis of ZLysONp has been found to be pH independent, within the experimental errors, for pH values higher than 6. In the pH range between 3 and 6, the value of K_s is pH dependent, changing from about 150 μM at neutral pH to 800 μM below pH 3 [see Tables I–III; for h.u. kallikrein, see Table I of Antonini et al. (1982)]. The data correspond to a simple titration curve with a pK_a value of 4.15 ± 0.1 (Figure 3).

The value of K_s (≃700 μM) is pH independent, over the same pH range explored (pH 3–6), for b. α-chymotrypsin (see Table IV and Figure 3).

Values of k_2/K_s, determined at different pH values, from experiments where $[E_0] \gg [S_0]$, are reported in Figure 1 for comparison with values of k_{cat}/K_m determined from steady-state experiments.

The pH dependence of k_3 for the b. β-trypsin and b. thrombin catalyzed hydrolysis of ZLysONp is reported in

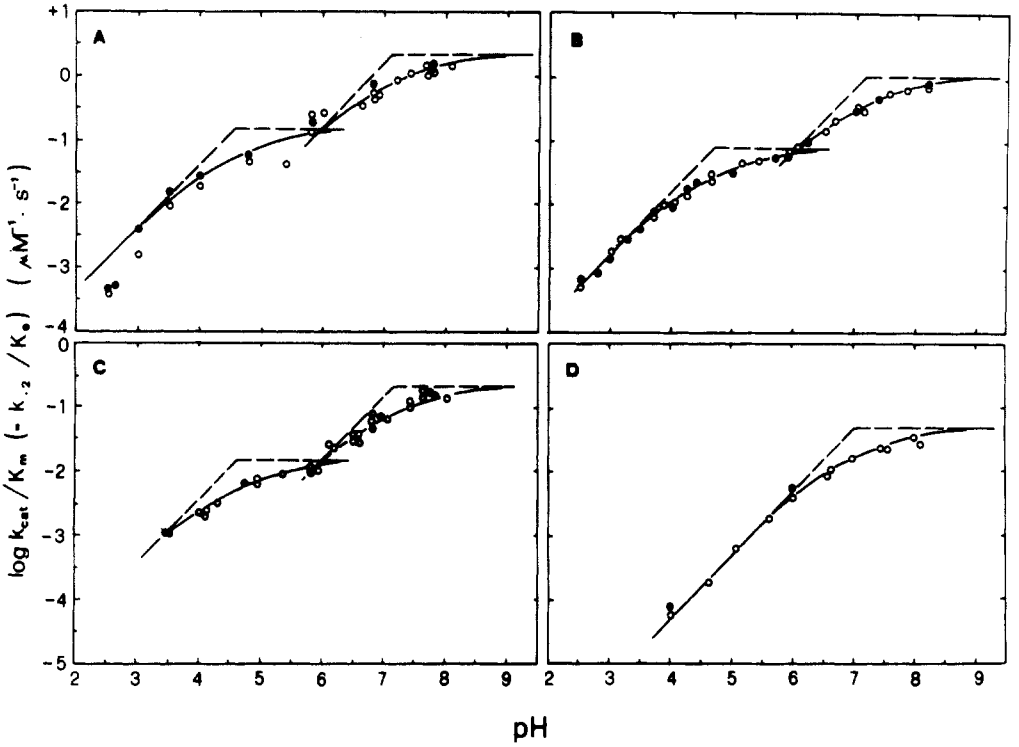

FIGURE 1: pH dependence of log k_{cat}/K_m (open symbols) for the b. β-trypsin (A), b. thrombin (B), h. urokinase (C), and b. α-chymotrypsin (D) catalyzed hydrolysis of ZLysONp. Filled symbols indicate values of log k_2/K_s calculated from pre-steady-state measurements. In the case of b. β-trypsin, b. thrombin, and h. urokinase, the fit of data for log k_{cat}/K_m ($=k_2/K_s$) with two pH transitions yields average pK_a values of 4.50 and 7.00. In the case of b. α-chymotrypsin, data for log k_{cat}/K_m ($=k_2/K_s$) may be fitted to a simple titration curve with a pK_a value of 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, $T = 21 \pm 1$ °C.

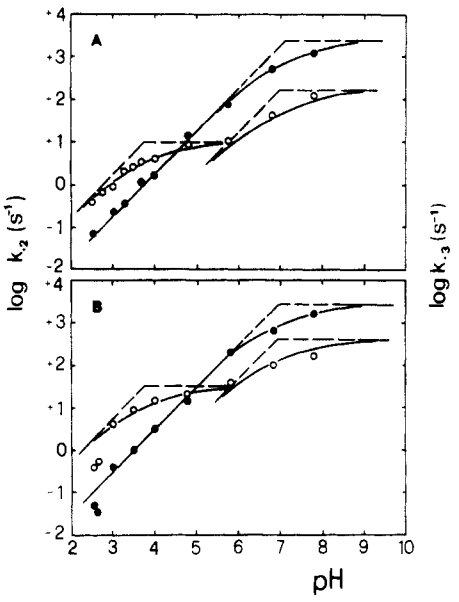

FIGURE 2: pH dependence of log k_2 (○) and log k_3 (●) values for the b. thrombin (A) and b. β-trypsin (B) catalyzed hydrolysis of ZLysONp. The fit of data for log k_2 with two pH transitions yields average pK_a values of 3.75 and 7.00. The data for log k_3 may be fitted to a simple titration curve with an average pK_a value of 7.10. For experimental conditions, see Figure 1.

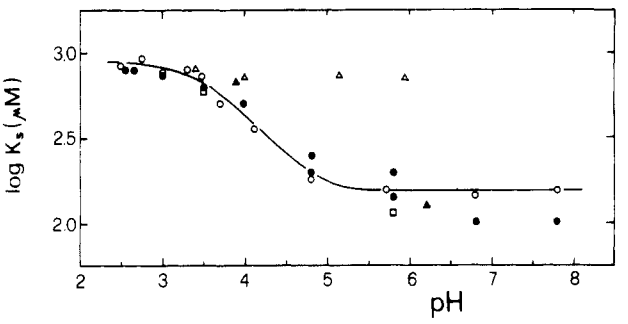

FIGURE 3: pH dependence of log K_s for the b. thrombin (○) catalyzed hydrolysis of ZLysONp. The solid line is the theoretical curve for one ionizable group with a pK_a value of 4.15. Values of K_s for b. β-trypsin (●), b. α-chymotrypsin (△), h. urokinase (□), and h.u. kallikrein (▲) catalyzed hydrolysis of ZLysONp are reported for comparison. For experimentals conditions, see Figure 1.

Figure 2. The data conform to a single ionizing group with a pK_a value of 7.1 ± 0.1.

As previously reported, at pH values where $k_2 \gg k_3$, 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) has been detected [see Tables I–III; for h.u. kallikrein, see Table I of Antonini et al. (1982)]. At pH values where k_2 is rate limiting in catalysis ($k_2 \ll k_3$), no burst phase is present [see Tables I–IV; for h.u. kallikrein, see Table I of Antonini et al. (1982)].

Steady-State Parameters of the Bovine β-Trypsin, Bovine Thrombin, Bovine α-Chymotrypsin, Human Urokinase, and Human Urinary Kallikrein Catalyzed Hydrolysis of Bz-ArgOEt, BzArgOMe, TosArgOMe, TosLysOMe, AcArgOMe, AcLysOMe, BzArgNHNp, BzLysNHNp, ZAlaONp, Bz-AlaOMe, AcAlaOMe, TosTyrOBe, ZTyrONp, BzTyrOEt, AcTyrOEt, and AcTyrNHNp. The dependence of initial velocity on the concentration of the various substrates for all the enzymes examined follows simple Michaelis–Menten kinetics.

Table V shows the values of k_{cat} and K_m for the esterase and amidase activity of the various enzymes. The choice of pH 6.8 is due to the instability of some substrates examined (notably ZLysONp, ZAlaONp, and ZTyrONp) at pH >7. The values of k_{cat} and K_m reported in this table are in quite agreement with those reported by other authors, if the dif-

Table V: Values of Steady-State Parameters for Enzymatic Hydrolysis of Cationic and Neutral Substrates at pH 6.8 (Phosphate Buffer 0.1 M, $T = 21 \pm 1$ °C)[a]

	b. β-trypsin		h. urokinase		h.u. kallikrein		b. thrombin		b. α-chymotrypsin	
substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)
BzArgOMe	14.0	280	0.0040	20000	5.5	120	12.0	280	0.1	8000
TosArgOMe	10.0	300	0.0038	18000	5.0	100	10.0	350	0.05	6200
BzArgOEt	11.0	300	0.0032	16000	4.8	120	14.0	260	0.08	9000
TosLysOMe	10.0	320	0.05	2000	1.2	380	4.0	620	0.05	9000
AcArgOMe	10.0	280	0.004	22000	5.2	110	15.0	300	0.06	12000
AcLysOMe	9.0	320	0.07	1500	1.2	420	3.0	650	0.05	10000
BzArgNHNp	0.7	300	0.0007	20000	0.5	100	0.62	280	0.004	12000
BzLysNHNp	0.8	280	0.006	2000	0.12	380	0.2	600	0.003	10500
ZLysONp	70.0	100	7.0	100	26.0	80	38.0	135	12.5	500
ZAlaONp	1.5	80	0.08	70	0.32	100	0.9	105	0.8	90
BzAlaOMe	0.2	240	0.01	280	0.030	300	0.15	250	0.06	320
AcAlaOMe	0.3	280	0.008	260	0.025	270	0.15	200	0.04	250
TosTyrOBe	1.2	700	0.001	14000	0.0015	6400	1.0	750	18.0	400
ZTyrONp	20.0	120	0.02	170	0.03	120	12.0	90	85.0	80
BzTyrOEt	1.8	600	0.001	16000	0.002	6000	0.8	900	15.0	380
AcTyrOEt	1.5	650	0.001	10000	0.002	5000	0.75	830	16.0	450
AcTyrNHNp	0.2	950	0.0002	20000	0.0003	12000	0.09	920	2.8	500

[a] At pH 8, k_{cat} for the b. β-trypsin catalyzed hydrolysis of TosArgOMe is about 5-fold larger than that of BzArgOEt (Walsh, 1970); however, since the pH dependence of k_{cat} is different in the two cases, the values of k_{cat} are about the same at pH 6.8.

ferences in temperature, pH, and buffer composition are taken into consideration.

The values of K_m have been found to be pH independent in the pH range explored (pH 3–8) for all substrates. The values of k_{cat}/K_m (data not shown) for b. β-trypsin, h. urokinase, h.u. kallikrein, and b. thrombin catalyzed hydrolysis of cationic substrates change with pH and may be fitted with the acidic and neutral transitions ($pK_a \simeq 4.5$ and $pK_a \simeq 7$) observed for the enzymatic hydrolysis of ZLysONp; those for the hydrolysis of neutral substrates show only the neutral transition ($pK_a \simeq 7$).

The values of k_{cat}/K_m for b. α-chymotrypsin catalyzed hydrolysis of both neutral and cationic substrates have the same pH dependence corresponding to a transition of $pK_a \simeq 7$. Inspection of Table V shows that the catalytic parameters for the same substrates vary largely, reflecting the different specificity profiles of the various enzymes. Substitutions at the N^{α} position on esters and anilides do not affect the steady-state parameters.

Effect of Acetamidine and Ethylamine on the Enzymatic Hydrolysis of ZAlaONp. The effect of adding acetamidine or ethylamine on the enzymatic hydrolysis of the neutral substrate ZAlaONp is reported in Tables I–IV [for h.u. kallikrein, see Table I of Antonini et al. (1982)]. The results clearly indicate a correlation between the substrate specificity of the enzymes and the effect of the two different promoters on the catalytic activity toward a neutral substrate.

The dissociation constants for binding of ethylamine and acetamidine to h.u. kallikrein, h. urokinase, b. β-trypsin, and b. thrombin vary with pH, with a pK_a of about 4.1 (see Figure 4).

The pH dependencies of the pre-steady-state parameters for the hydrolysis of ZAlaONp by b. β-trypsin, b. thrombin, h.u. kallikrein, and h. urokinase in the presence of saturating levels of ethylamine and acetamidine show that at acid pH values (pH ≤4) the k_3 step is rate limiting in catalysis, whereas at pH ≥6, the k_2 step becomes rate limiting [see Tables I–III; for h.u. kallikrein, see Table I of Antonini et al. (1982)]. On the other hand, in the absence of ethylamine and acetamidine, the acylation step (k_2) is rate limiting in the enzymatic hydrolysis of ZAlaONp, over the whole pH range explored.

In contrast, b. α-chymotrypsin catalyzed hydrolysis of ZAlaONp is not affected by the presence of ethylamine and acetamidine (see Table IV). Furthermore for b. α-chymotrypsin, over the pH range 3–6, k_2 remains the rate-limiting step for the enzymatic hydrolysis of ZLysONp and ZAlaONp in the presence or absence of saturating levels of ethylamine and acetamidine, and the values of K_s, in all cases, are pH independent.

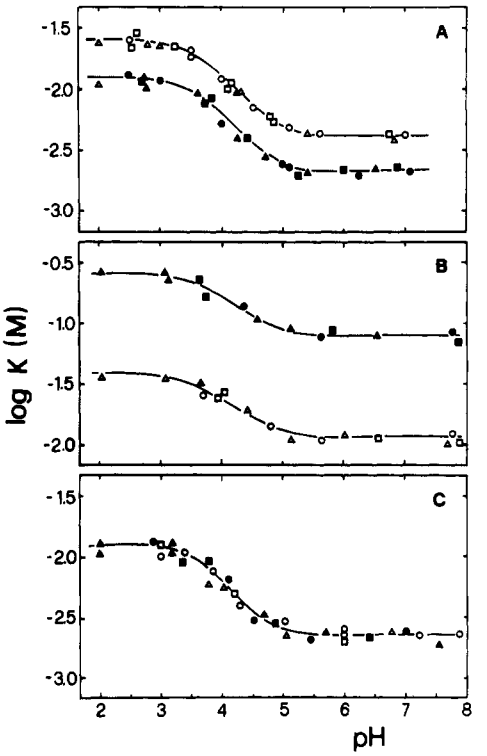

FIGURE 4: pH dependencies of the dissociation constant for the reaction of ethylamine (open symbols) and acetamidine (filled symbols) with b. thrombin (A), h. urokinase (B) and b. β-trypsin (C). The data have been obtained (i) by the promoter effect of cations on the enzymatic hydrolysis of ZAlaONp (circles), (ii) by the inhibition effect on the hydrolysis of ZLysONp (squares), and (iii) by spectrophotometric titrations (triangles). The data may be fitted with a simple pH transition with an average pK_a value of 4.10. For experimental conditions, see Figure 1.

No formation of the amide derivatives has been observed in the case of enzymatic catalysis by h. urokinase and b. thrombin of the hydrolysis of ZLysONp and ZAlaONp in the

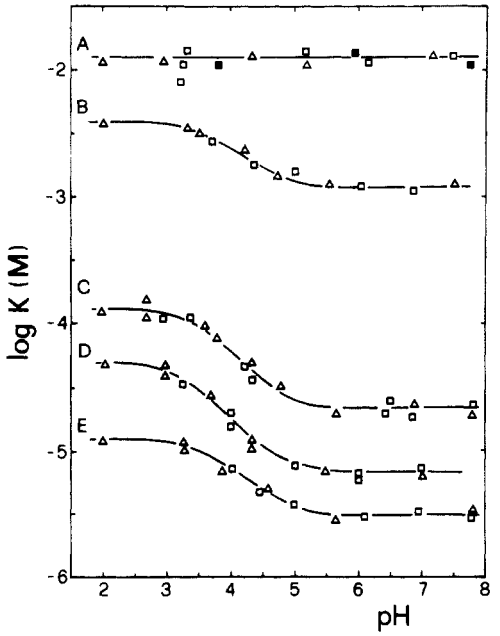

FIGURE 5: pH dependencies of the dissociation constant for the reaction of benzamidine with b. α-chymotrypsin (A), h. urokinase (B), b. β-trypsin (C), b. thrombin (D), and h.u. kallikrein (E). The data have been obtained (i) by the inhibition effect on the enzymatic hydrolysis of ZLysONp (□) and ZTyrONp (■) and (ii) by spectrophotometric titrations (△). The data for h. urokinase, b. β-trypsin, b. thrombin, and h.u. kallikrein may be fitted with a simple pH transition with an average pK_a value of 4.10. For experimental conditions, see Figure 1.

presence of ethylamine and acetamidine, as already reported for b. β-trypsin, b. α-chymotrypsin (Antonini & Ascenzi, 1981) and h.u. kallikrein (Antonini et al., 1982).

Inhibition by Benzamidine of the Enzymatic Hydrolysis of ZLysONp and ZTyrONp. For independent verification of the differences in the binding properties of the recognition site of b. β-trypsin, b. α-chymotrypsin, h. urokinase, h.u. kallikrein, and b. thrombin, the inhibitory effect of benzamidine on the enzymatic hydrolysis of ZLysONp and ZTyrONp was investigated.

Figure 5 shows the effect of pH on the inhibition constant of benzamidine on the catalyzed hydrolysis of ZLysONp and ZTyrONp. Values of the dissociation constant evaluated by spectrophotometric titrations are also reported for comparison in Figure 5.

The pH dependencies of the dissociation and inhibition constants for the binding of benzamidine to h.u. kallikrein, b. β-trypsin, h. urokinase, and b. thrombin, all acting on cationic substrates, may be fitted with a simple pH transition with an average pK_a value of 4.1. No pH dependence of the value of the inhibition constant for b. α-chymotrypsin was observed.

In agreement with the high specificity toward the guanidinium group (see Figure 4), h.u. kallikrein and b. thrombin show a higher affinity for benzamidine than b. β-trypsin and h. urokinase.

Spectral Properties of the Enzymes in the Absence and Presence of Cationic Ligands. It is known that trypsin undergoes a reversible acid–alkaline transition between pH 2.5 and 8 directly detectable in the ultraviolet region (East & Trowbridge, 1968). On the other hand, the absorption spectrum of the trypsin–benzamidine complex is pH independent (East & Trowbridge, 1968).

It appeared therefore of interest to investigate the pH dependence of the absorption spectra of the free proteases examined and of their complexes with benzamidine, ethylamine, and acetamidine.

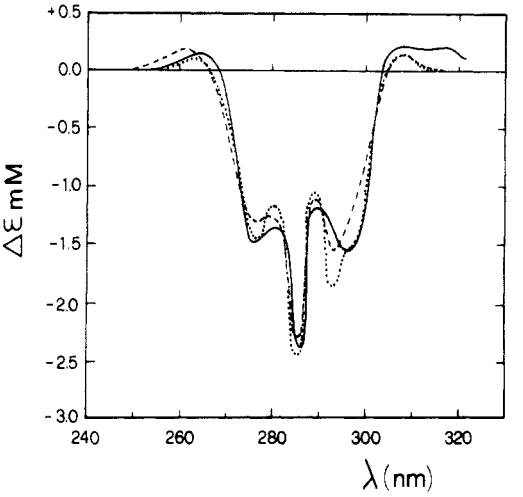

FIGURE 6: pH difference spectra of b. β-trypsin (- - -), b. thrombin (—), and h. urokinase (···). The reference solution was at pH 7.5, and the sample solution was at pH 2.7. The data have been obtained in phosphate buffers all 0.1 M, T = 21 ± 1 °C.

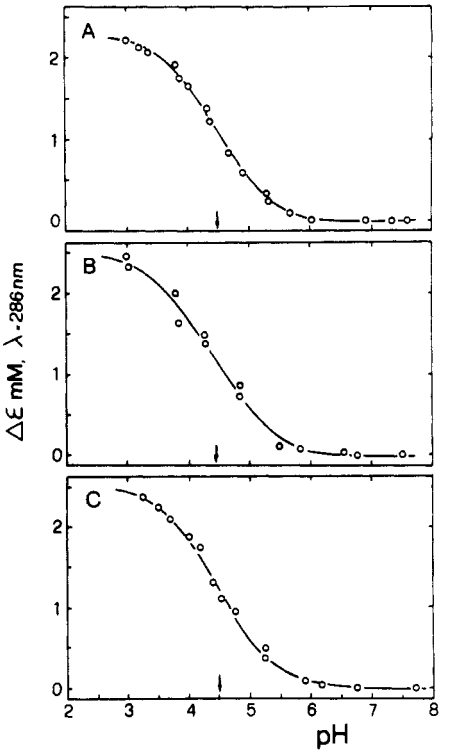

FIGURE 7: pH titrations of b. thrombin (A), h. urokinase (B), and b. β-trypsin (C). The fit of data with a simple pH transition yields an average pK_a value of 4.5. For experimental conditions, see Materials and Methods.

Figure 6 shows the pH difference spectra of b. β-trypsin, h. urokinase, and b. thrombin between 230 and 330 nm. The pH dependence of the spectral properties of the free enzymes reflects the ionization of a group with an average pK_a value of 4.50 ± 0.1 (see Figure 7). Also h.u. kallikrein undergoes a closely similar spectral change, showing a pK_a value of 4.45 ± 0.1 (Antonini et al., 1982). Moreover, as previously reported for h.u. kallikrein, no pH difference spectra are present between 230 and 260 nm. Over this wavelength range, absorption changes on binding ethylamine, acetamidine, and benzamidine are present, corresponding to a difference spectrum with a maximum at 240–245 nm and a $\Delta\epsilon$ (mM) of about 2 (East & Trowbridge, 1968; Antonini et al., 1982). These changes are pH independent.

On the other hand, the absorption spectra of free b. α-chymotrypsin and of the benzamidine-bound derivative are pH independent in the explored wavelength range (230–330 nm). Moreover, in agreement with previous results (Antonini & Ascenzi, 1981), no spectral changes reflecting the binding of ethylamine and acetamidine to b. α-chymotrypsin have been observed.

Discussion

This paper provides a comparison of the catalytic properties of h.u. kallikrein, b. β-trypsin, h. urokinase, b. thrombin, and b. α-chymotrypsin. With regard to substrate specificity, the arginine ester or anilide derivatives are more sensitive substrates for h.u. kallikrein and b. thrombin than the corresponding ones of lysine. The greater rate of catalysis is reflected in higher values of k_{cat} and lower K_m. It is noteworthy that h.u. kallikrein and b. thrombin differ widely from one another in the ability to catalyze the hydrolysis of substrates containing aromatic side chains, b. thrombin being orders of magnitude more efficient than h.u. kallikrein. The catalytic parameters for b. β-trypsin catalyzed hydrolysis of substrates containing arginine and lysine are undistinguishable. On the other hand, h. urokinase shows more favorable steady-state parameters in the hydrolysis of lysine rather than arginine derivatives.

The differences in specificity among the various enzymes are also brought out very clearly by the different promoter effect of acetamidine and ethylamine in the hydrolysis of noncationic substrates. Bovine α-chymotrypsin shows no preference for cationic substrates, and its catalysis is not promoted by acetamidine or ethylamine. Thus, b. β-trypsin, h.u. kallikrein, h. urokinase, and b. thrombin, although all acting on cationic substrates, show striking variations in specificity which may be related to subtle changes in the stereochemical structure of the specificity determining site (Christova et al., 1980).

It was reported before for b. β-trypsin (Antonini & Ascenzi, 1981) and h.u. kallikrein (Antonini et al., 1982) that the rate-limiting step in the catalytic hydrolysis of ZLysONp and ZAlaONp in the presence of cationic ligands changes with pH. This can now be generalized also for h. urokinase and b. thrombin. For all these enzymes and human thrombin (Kézdy et al., 1965), k_3 is rate limiting in catalysis at pH ≤4 whereas, at pH ≥6, k_2 becomes rate limiting. On the other hand, the rate-limiting step (k_2) for the b. α-chymotrypsin catalyzed hydrolysis of both ZLysONp and ZAlaONp does not change with pH.

The pH dependence of the ratio k_{cat}/K_m ($=k_2/K_s$) for the h.u. kallikrein [see Antonini et al. (1982)], b. β-trypsin, h. urokinase, and b. thrombin catalyzed hydrolysis of ZLysONp indicates [according to Peller & Alberty (1959)] that the free form of the enzymes undergoes two pH transitions with pK_a values of about 4.5 and 7.0 (see Figure 1). On the other hand, b. α-chymotrypsin undergoes only one acid–alkaline transition with a pK_a value of 7.0 ± 0.1 (see Figure 1).

The increase of the value of K_s for the b. β-trypsin, h. urokinase, h.u. kallikrein, and b. thrombin catalyzed hydrolysis of ZLysONp, from pH 6 to 4, reflects, according to linkage relations (Wyman, 1964), an acid shift in the pK_a value of the acidic group ($pK_a \simeq 4.5$ in free enzymes) on binding ZLysONp amounting to about 0.8 unit.

In addition, the effect of pH on the dissociation constant for the binding of ethylamine, acetamidine, and benzamidine to b. β-trypsin, h. urokinase, h.u. kallikrein, and b. thrombin implies a pK_a shift from 4.5 in free enzymes to 3.7 in the complexes. Thus, also the pH dependence in the acid region of the affinity for cationic substrates or ligands which was observed before for b. β-trypsin (Antonini & Ascenzi, 1981) and h.u. kallikrein [see Antonini et al. (1982)] appears to be a common feature of other serine proteases acting on cationic substrates.

The absorption spectra of the free form of enzymes acting on cationic substrates change with pH according to a simple titration curve with an average pK_a value of 4.5. On the other hand the absorption spectra of the ligand-bound enzymes are pH independent. The difference absorption spectra of free and ligand-bound b. β-trypsin, h. urokinase, h.u. kallikrein, and b. thrombin are also similar (see Figure 6).

In conclusion, the results of this and of the previous papers (Antonini & Ascenzi, 1981; Antonini et al., 1982; Ascenzi et al., 1981) indicate that serine proteases acting on cationic substrates, notably b. β-trypsin, b. thrombin, h.u. kallikrein, and h. urokinase, contain a similarly located ionizable residue, of $pK_a = 4.50 \pm 0.1$ in the free enzymes, which affects the binding of cationic substrates or ligands, the spectral properties, and the rate-limiting step in catalysis. Such a residue should be absent in b. α-chymotrypsin. From the amino acid sequences (Walsh, 1970; Wilcox, 1970) and from the three-dimensional structures of b. β-trypsin (Huber et al., 1974; Bode & Schwager, 1975) and b. α-chymotrypsin (Blow, 1971; Birktoft & Blow, 1972), the residue may be identified as aspartate-189 in b. β-trypsin, which in b. α-chymotrypsin is replaced by a serine.

Added in Proof

While this paper was in preparation, N^{α}-carbobenzoxy-L-arginine *p*-nitrophenyl ester (ZArgONp) was synthesized [according to Glass & Pelzig (1978)]. Catalytic parameters for the enzymatic hydrolysis of ZArgONp by b. β-trypsin ($k_{cat} = 70\ s^{-1}$; $K_m = 100\ \mu M$), h. urokinase ($k_{cat} = 0.74\ s^{-1}$; $K_m = 526\ \mu M$), h.u. kallikrein ($k_{cat} = 66.7\ s^{-1}$; $K_m = 51.5\ \mu M$), b. thrombin ($k_{cat} = 91\ s^{-1}$; $K_m = 87\ \mu M$), and b. α-chymotrypsin ($k_{cat} = 12.5\ s^{-1}$; $K_m = 500\ \mu M$) were evaluated at pH 6.80 (0.1 M phosphate buffer, $T = 21$ °C). The comparison of the values of catalytic parameters for the enzymatic hydrolysis of ZArgONp and ZLysONp (see Table V) reinforces the differences in the specificity between the serine proteases examined.

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Studies on the Chirality of Sulfoxidation Catalyzed by Bacterial Flavoenzyme Cyclohexanone Monooxygenase and Hog Liver Flavin Adenine Dinucleotide Containing Monooxygenase†

David R. Light,‡ David J. Waxman,§ and Christopher Walsh*

ABSTRACT: The stereochemical outcome of oxygen transfer to the sulfur moiety of aryl alkyl sulfides catalyzed by two flavoenzyme monooxygenases has been determined by resolution of sulfoxide product enantiomers on a high-pressure liquid chromatography column [Pirkle, W. H., Finn, J. M., Schreiner, J. L., & Hamper, B. C. (1981) *J. Am. Chem. Soc. 103*, 3964–3966] containing a 3,5-dinitrobenzoyl-D-phenylglycine chiral stationary phase. With 4-tolyl ethyl sulfide as substrate, cyclohexanone monooxygenase from *Acinetobacter* produces predominantly the (*S*)-(−)-sulfoxide (82% *S*, 18% *R*), a modest enantioselectivity. In contrast, the flavin adenine dinucleotide (FAD) containing monooxygenase purified from hog liver microsomes carries out sulfoxidation to yield the (*R*)-(+)-sulfoxide enantiomer as major product (95% *R*, 5% *S*). The presence of the minor sulfoxide enantiomer in each case appears to be due to incomplete chiral processing by each enzyme and not to a competing, achiral, nonenzymic sulfoxidation process. The mammalian FAD-containing monooxygenase also oxygenates the divalent sulfur of the antiarthritic drug sulindac sulfide to yield a single dextrorotatory isomer of the sulfoxide prodrug. Analysis of the chiral outcome of sulfoxidation catalyzed by rat liver microsomes indicated that phenobarbital treatment increases the capacity for *S*-(−)-oxygenation of 4-tolyl ethyl sulfide, suggesting that the phenobarbital-induced cytochrome P-450 isozymes catalyze formation of the (*S*)-(−)-sulfoxide preferentially, a surmise validated in the following paper [Waxman, D. J., Light, D. R., & Walsh, C. (1982) *Biochemistry* (following paper in this issue)]. With sulindac sulfide as substrate, though, both control and phenobarbital-induced microsomes catalyze sulfoxidation to yield the same (+)-sulfoxide enantiomer generated by the purified FAD-containing monoxygenase, suggesting a low degree of participation by the cytochrome P-450 isozymes in sulfoxidation of this compound.

The flavin adenine dinucleotide (FAD)[1] containing monooxygenase (EC 1.14.13.8, *N*,*N*-dimethylaniline monooxygenase), first characterized by Ziegler and co-workers in liver microsomes (Machinist et al., 1968; Ziegler, 1980), catalyzes oxygen transfer to nitrogen and sulfur atoms in many substrate types and is, along with the cytochrome P-450 mo-

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[1] Abbreviations: FAD, flavin adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; HPLC, high-pressure liquid chromatography; GC/MS, gas chromatography/mass spectroscopy; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NMR, nuclear magnetic resonance; Me_3Si, trimethylsilyl.

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