BBA 65884

BINDING OF COMPETITIVE INHIBITORS TO THE DIFFERENT pH-DEPENDENT FORMS OF TRYPSIN*

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(Received December 9th, 1968)

SUMMARY

Some of the rotatory, spectral, ionic and catalytic properties of trypsin (EC 3.4.4.4) have been studied, in the pH range from 1.5 to 12.5, in the presence and absence of synthetic competitive inhibitors. In the alkaline and acidic pH ranges where trypsin is reversibly denatured, the formation of the trypsin–inhibitor complex protects the enzyme against transconformation; at neutral pH, where trypsin is in the native form, the complex formation sometimes induces a change in the enzyme structure.

In the alkaline pH range (a) the rotatory dispersion curve of the complex is pH independent; (b) the formation of the complex induces a decrease of the enzyme absorbance; (c) the formation of the complex produces a proton uptake by the enzyme; (d) the affinity of inhibitors decreases when the pH increases. All these results can be related to the ionization of an enzyme group having an apparent pK of about 10.

In the acidic pH range (a) the rotatory power of the complex is pH independent, and (b) the complex formation produces a proton release by the enzyme. The changes in the ionic and rotatory properties of trypsin as a function of inhibitor concentration have allowed the evaluation of the dissociation constant of the benzamidine–trypsin complex between pH 4.5 and 1.5. A scheme for the interaction between the inhibitor and the different pH dependent forms of trypsin is proposed: it accounts for the pH dependence of the complex dissociation constant and also for the dependence of the number of protons released by the enzyme. The lowering of the inhibitor affinity in the acidic pH range is due to the protonation of two enzyme groups, one having an apparent pK of 3.7, the other a true pK of 4.5.

At neutral pH, the enzyme–inhibitor complex formation may induce a change in both the enzyme and the inhibitor structure; formation of the trypsin–proflavin complex, for instance, makes the inhibitor optically active.

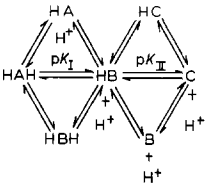
Abbreviations: BA, *N*-α-benzoyl-L-arginine; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; APME, *N*-α-acetyl-L-phenylalanine methyl ester; TAME, *N*-α-*p*-toluenesulfonyl-L-arginine methyl ester.

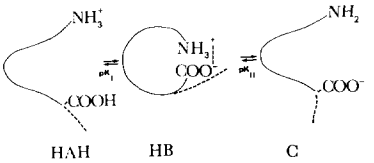
* A preliminary report of this work was presented at the 5th Meeting of the Federation of European Biochemical Societies, Prague, July 1968, No. 742.

INTRODUCTION

In an earlier report[1], it has been shown that the binding of low molecular weight competitive inhibitors changes the polarimetric properties of trypsin (EC 3.4.4.4). This phenomenon has been studied at different pH values. Between pH 5 and 8.5, there is no or only a small variation in $[\alpha]$; in the acidic or alkaline pH ranges, however, where trypsin is reversibly denatured, the complex formation induces a decrease in the levorotation of the enzyme. The new specific rotation has a value similar to that observed at neutral pH.

In the pH range from 2 to 11, free enzyme exists in the following states indicated in Scheme 1.





Scheme 1. HAH, HB and C represent three main forms of trypsin at acidic, neutral and alkaline pH, respectively.

Scheme 2. Hypothetical scheme for the conformational changes in trypsin as a function of pH. See explanations in text.

The equilibrium

$$HAH \overset{K_I}{\rightleftharpoons} HB + H^+$$

is controlled by an enzymic group having an apparent pK_I of 3.7. The other equilibrium,

$$HB \overset{K_{II}}{\rightleftharpoons} C + H^+,$$

is controlled by an ionic group of apparent pK_{II} about 10. The nature of these groups is not yet known. Hypothetically, it may be suggested that, as in chymotrypsin[2–6], the acidic group is the carboxylic group of an aspartic acid (Asp-182 near to active serine), and the basic group the NH_2-terminal isoleucyl α-amino group. Recently it has been suggested that this α-amino group is involved in the activity of trypsin[7,26].

The form HB is the most compact: a part of the polypeptidic chain must be strained in an active conformation by a non-covalent bond between these two groups, according to Scheme 2. The bond release, following the basic group deprotonation in the alkaline pH range or the carboxylic group protonation in the acidic pH range, induces a protein unfolding accompanied by the formation of species C and HAH, respectively, whose polarimetric properties are similar.

In the presence of excess inhibitor (or substrate), the enzyme's levorotation remains constant at all pH values. If the native form HB binds the inhibitor preferentially, there will be a decrease in the inhibitor's affinity at pH > 9 and < 4.5. Consequently, the protonic equilibrium will be different in the free enzyme and in the enzyme–inhibitor complex: pK_I will decrease and conversely pK_{II} will increase.

Moreover, in the acidic pH range, there is a decrease in the inhibitor's affinity due to the protonation of another carboxylic group of the enzyme. The anionic form of this carboxylic group interacts electrostatically with the positive charge of the protonated inhibitor[1,8]. It has been suggested that this group could be the γ-carboxylic group of glutamic acid No. 173 (ref. 9) or the β-carboxylic group of aspartic acid No. 177 (ref. 27).

Some of the rotatory, spectral, ionic and catalytic properties of trypsin have been studied in the pH range 1.5–11.5, in the presence and in the absence of competitive inhibitors. Some results have already been reported[1]. Complementary results, mainly obtained with stronger inhibitors (benzamidine or proflavin), are given in this paper.

MATERIALS AND METHODS

Materials and experimental procedures are similar to those described in an earlier paper[1]. In addition to butylamine, benzylamine and *N*-α-benzoyl-L-arginine (BA), we have used benzamidine[10] recrystallized from a water–ethanol mixture (Aldrich Chemical Co.) (m.p., 163°), and proflavin dichlorhydrate (Mann Research Laboratory). The ionization pK of benzamidine, evaluated by potentiometric measurements, is 11.8 $\pm$ 0.2, at 15°; the enzyme–inhibitor dissociation constant, determined by inhibition of the hydrolysis of *N*-α-acetyl-L-phenylalanine methyl ester (APME), is $2.7 \cdot 10^{-5}$ M at 15° and pH 7. The concentration of proflavin was measured spectrophotometrically (ε at 444 mμ = $3.9 \cdot 10^{4}$)[11]; its competitive inhibition constant is $4 \cdot 10^{-5}$ M at neutral pH and 25° (refs. 12, 13). *N*-α-Acetyl-L-tyrosine amide is a product of the Sigma Chemical Co., and APME, a product of the Mann Research Laboratory; both substances were used without further purification.

Once-crystallized trypsinogen, having less than 1% tryptic activity (as measured with *N*-α-benzoyl-L-arginine ethyl ester (BAEE)), was purchased from the Worthington Biochemical Corp. Its concentration was determined by the absorbance at 280 mμ ($E^{1\,cm}_{1\,mg/ml} = 1.54$).

Optical rotatory dispersions were measured with the Spectropol I Fica spectropolarimeter, and spectral data were obtained with Cary model 14 or 15 recording spectrophotometers.

Changes in pH due to association of benzamidine and trypsin (or trypsinogen) were recorded with a Tacussel model EPL I linear potentiometric recorder coupled with the EIL vibron 33 B pH meter, the sensitivity of which is about 0.001 pH unit.

Experiments were carried out at 10 or 15°, and ionic strength, 0.3 (NaCl).

RESULTS AND DISCUSSION

1. *Experimental results in the neutral pH range*

At neutral pH, the binding of inhibitor to enzyme may induce some isomerization of the enzyme[14–18]. Hence in the presence of inhibitor, the form HB (Scheme 1) may be converted to a modified form HB′ or HB″, reflecting some direct interaction between the two molecules.

As shown in Table I, the binding of low molecular weight competitive inhibitors to trypsin induces a decrease in the levorotation of the enzyme. This effect depends on the inhibitor's nature. Butylamine does not change the rotatory power of trypsin

TABLE I

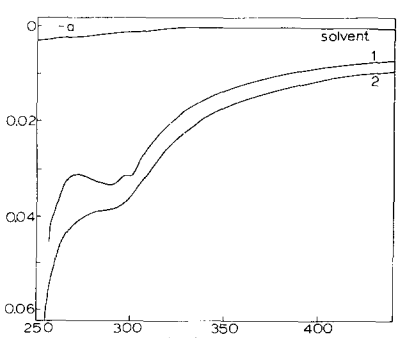
EXPERIMENTAL DATA FOR THE SPECIFIC ROTATORY POWER $[\alpha]_{395\ m\mu}$ AND DISSOCIATION CONSTANT OF DIFFERENT ENZYME–INHIBITOR COMPLEXES AT NEUTRAL pH (pH 6–8)

$[\alpha]^{E}_{395\ m\mu} = -96$. θ, 10°. Ionic strength, 0.3.

EI complex	K_i *(M)*	$[\alpha]^{EI}_{395\ m\mu}$	$\frac{[\alpha]^{E}_{395\ m\mu} - [\alpha]^{EI}_{395\ m\mu}}{[\alpha]^{E}_{395\ m\mu}} \times 100$ (%)
Trypsin–butylamine	$1.6\ (\pm 0.5) \cdot 10^{-3}$	−96	0
Trypsin–BA	$8\ (\pm 2) \cdot 10^{-4}$	−92	4
Trypsin–benzylamine	$3.3\ (\pm 0.8) \cdot 10^{-4}$	−89	7
Trypsin–benzamidine	$2.7\ (\pm 0.5) \cdot 10^{-5}$	−88	8

(Fig. 1, Curve 1), while the binding of an aromatic inhibitor, such as benzamidine, induces a significant lowering of the levorotation (Fig. 2). The larger the apparent affinity of inhibitor for trypsin, the larger is the observed effect within certain limits (Table I).

Some authors have shown that the absorbances of benzamidine (or derivatives of benzamidine) and of trypsin were modified in the enzyme–inhibitor complex[9,19]. Therefore some complementary adjustment between inhibitor and enzyme seems evident. Similarly a large change in the structure of a competitive inhibitor, proflavin, is found when it is bound to the enzyme. In Fig. 3, the rotatory dispersion of trypsin in the presence and absence of non-optically active proflavin is shown. A positive Cotton effect at 458 mμ appears in the absorption band of bound proflavin (the absorption maximum of the chymotrypsin–proflavin complex is also at 458 mμ (ref. 20)); its amplitude is dependent on inhibitor concentration. The positive maximum is at 478 mμ ($[M]_{478 m\mu} = +320°$) and the negative minimum at 428 mμ ($[M]_{428 m\mu} = -250°$). Therefore, in the trypsin–proflavin complex, the inhibitor is in an asymmetrical configuration and becomes optically active.



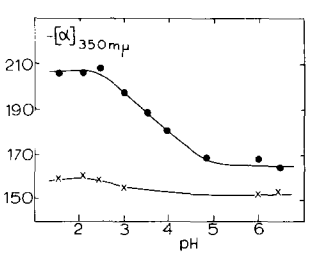
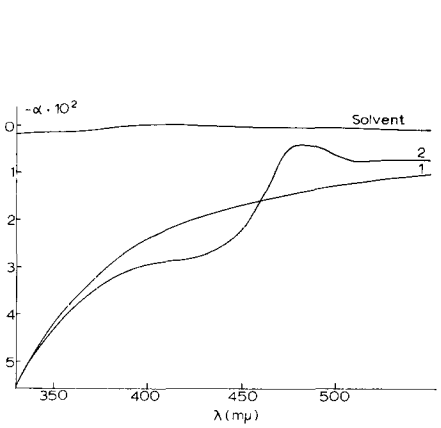


Fig. 1. Optical rotatory dispersions of trypsin and trypsin–butylamine complex. Curve 1, free enzyme at pH 6.85; enzyme–butylamine complex at pH 6.85 and pH 10.45. Curve 2, free enzyme at pH 10.45. Experimental conditions: θ, 11°; ionic strength, 0.3; [enzyme], 0.8 mg/ml = $3.4 \cdot 10^{-5}$ M; [butylamine], $8.5 \cdot 10^{-2}$ M; optical path, 0.1 dm.

Fig. 2. Effect of pH on the specific rotatory power of free trypsin (●) and of trypsin–benzamidine complex (×) in the neutral and acidic pH range. Experimental conditions: θ, 15°; ionic strength, 0.3; [enzyme], 3.1 mg/ml; λ, 350 mμ; optical path, 0.2 dm.



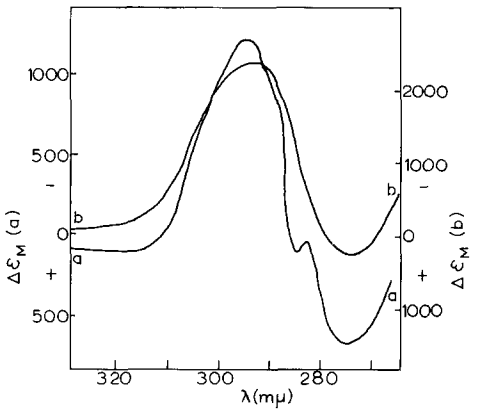


Fig. 3. Optical rotatory dispersions of trypsin in absence (Curve 1) and in presence (Curve 2) of proflavin. Experimental conditions: θ, 10°; ionic strength, 0.3; pH 5.7; [enzyme], 4.8 mg/ml = $2 \cdot 10^{-4}$ M; [proflavin], $8.1 \cdot 10^{-5}$ M; optical path, 0.05 dm.

Fig. 4. Spectrum a. Difference spectrum of trypsin in the presence (sample cell) and in the absence (reference cell) of butylamine 0.17 M, at pH 11.0. Experimental conditions: θ, 10°; ionic strength, 0.3; [enzyme], 0.49 mg/ml = $2.1 \cdot 10^{-5}$ M. Spectrum b. Difference spectrum of 10^{-5} M *N*-α-acetyl-L-tyrosine amide, at pH 7 (sample cell) and in 10^{-2} M NaOH (reference cell). The $\Delta\varepsilon_{293\ m\mu}$ experimental value is 2400, in good agreement with the value given by MARTIN *et al.*[21].

2. Experimental results in the alkaline pH range

Comparative rotatory dispersions of free enzyme and trypsin–butylamine complex at pH 10.45

As previously shown[1], the optical rotation at 436 mμ of the trypsin–butylamine complex, between pH 7 and 11, is essentially the same as that of the free enzyme at neutral pH. Now the same result is obtained at other wavelengths, between 450 and 250 mμ (Fig. 1, Curve 1).

At pH 10.45, the levorotation of the free enzyme increases compared with the levorotation observed at neutral pH, suggesting an unfolding of the protein (Fig. 1, Curve 2). The Cotton effects between 300 and 270 mμ in the aromatic groups absorbance range decrease markedly in intensity; this could be due to a partial or complete unmasking of one or several enzymic aromatic chromophores. At this pH, the rotatory dispersion of the enzyme–inhibitor complex is identical with that of the HB neutral species and the environment of the aromatic residues, as seen by Cotton effects, is therefore likely to be the same (Fig. 1, Curve 1). The binding of butylamine by trypsin displaces the equilibrium HB $\rightleftharpoons$ C + H^+ towards the form HB.

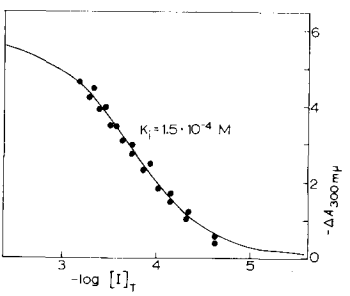
Comparative absorbances of free trypsin and trypsin–inhibitor complex in the alkaline pH range

It has been previously shown that the absorbance of the trypsin–butylamine complex, at 300 or 295 mμ, becomes lower in the alkaline pH range relative to that of the free enzyme[1].

The difference spectrum between the trypsin–butylamine complex and the free enzyme, at pH 11, is compared in Fig. 4 with the difference spectrum of *N*-α-tyrosine amide at neutral pH and in 10^{-2} M NaOH. Apparently, the addition of butylamine to trypsin at pH 11 induces partial or complete masking of one or several tyrosine groups

(an average of 0.5 is evaluated here), and a perturbation in the absorbance of some other aromatic chromophores (shoulder at 289 mμ, maxima at 284 and 274 mμ). Then, according to the rotatory dispersion results, the displacement of the equilibrium $HB \rightleftharpoons C + H^+$ to the form HB implicates the masking of one (or several) tyrosine group. In the species HB, this group is inside the molecule and not accessible to solvent; conversely, in the alkaline species C, this group is unmasked.

This spectral change allows one to evaluate the dissociation constant of the trypsin–benzamidine complex at pH 10.9 (Fig. 5). Benzamidine has been preferred



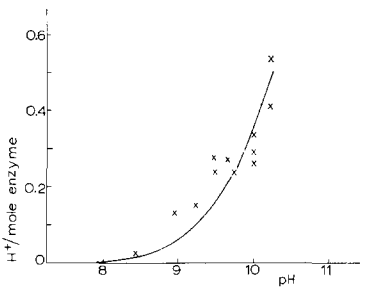


Fig. 5. Absorbance change of trypsin $\Delta A_{300\,m\mu}$ *versus* $-\log$ [benzamidine] at pH 10.9; ionic strength, 0.3; θ, 15°; [enzyme], $5.6 \cdot 10^{-5}$ M; absorbance scale, 0–0.1 (neutral filter in the reference beam). 1–25 μl of benzamidine ($6 \cdot 10^{-2}$ M) buffered solution were added to 2.5 ml of trypsin buffered solution. Experimental values are corrected for dilution effect and benzamidine's small absorbance. The curve is calculated for a value of $K_i = 1.5 \cdot 10^{-4}$ M.

Fig. 6. Effect of pH on the number of protons consumed by one enzyme molecule during trypsin–benzamidine interaction in the alkaline pH range. θ, 15°; ionic strength, 0.3; [enzyme], 10^{-4} M; [benzamidine], $7.5 \cdot 10^{-4}$ M. 10 μl of benzamidine solution (concn., 0.23 M) at a given pH are added to 3.1 ml of non-buffered trypsin solution at the same pH. Control measurements without trypsin were made at high pH where benzamidine begins to ionize. A second addition of benzamidine (10 μl) was made to verify that trypsin was saturated with inhibitor. The parameter H+/mole enzyme is calculated on the basis that only 83% of the enzyme molecules can bind the inhibitor (*p*-nitrophenylacetate titration).

because it is a better inhibitor than butylamine ($K_{i\ \text{benzamidine}} = 2.7 \cdot 10^{-5}$ M; $K_{i\ \text{butylamine}} = 1.6 \cdot 10^{-3}$ M, at neutral pH), and its own ionization pK is higher. At pH 10.9, the dissociation constant has been estimated to be $1.5 \cdot 10^{-4}$ M, while at pH 8.5 it is about 6 times smaller. This increase may be related to the ionization of a group on the enzyme having an apparent pK of about 10.

Proton uptake in the association of trypsin and benzamidine

At a given pH between 8.5 and 10.3 the association of benzamidine ($3.8 \cdot 10^{-3}$ M) and trypsin (10^{-4} M) in a non-buffered solution causes an alkalinization of the medium; the increase is 0.01–0.04 pH unit, depending on the initial pH. The semiquantitative titration of the proton uptake by 0.01 M HCl, shows that the number of protons consumed by the enzyme in the presence of excess benzamidine increases with pH, as indicated in Fig. 6.

The formation of trypsin–inhibitor complex induces a shift towards alkaline pH of the apparent ionization constant of one (or several) residues on the enzyme.

Tryptic hydrolysis of APME and BAEE, in the alkaline pH range

Inhibition by benzamidine. (*a*) The tryptic hydrolysis of a non-specific substrate,

APME, in the presence and absence of benzamidine was studied by the pH-stat method, at constant pH, between 8.5 and 10.3 (ionic strength = 0.1) (Fig. 7).

In the absence of inhibitor and under conditions where the substrate concentration ($[S] = 2 \cdot 10^{-3}$ M) is smaller than the Michaelis constant (K_m = approx. $5 \cdot 10^{-2}$ M), the initial velocity decreases as the pH increases, according to the ionization of a group on the trypsin having a pK of 10 (ref. 22). This velocity is given by the following relationship:

$$v_0 = \frac{V \cdot S}{K_m(1 + K_a/H)}$$

In the presence of benzamidine ($[I] = 5.6 \cdot 10^{-5}$ M), the initial reaction velocity becomes:

$$v_i = \frac{V \cdot S}{K_m(1 + K_a/H)(1 + I/K_{i(pH)})}$$

The constant $K_{i(pH)}$ is the assumed pH dependent inhibition constant.

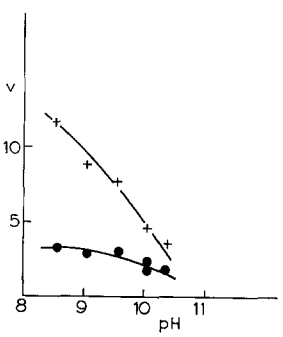
One may write:

$$v_i/v_0 = \frac{1}{1 + I/K_{i(pH)}}$$

As the fraction of ionized benzamidine is negligible in this pH range, owing to its high ionization pK (pK = 11.8 ± 0.2 at 15°), any variation of the ratio v_i/v_0 with pH indicates that the inhibition constant K_i is pH dependent. A theoretical curve calculated from the expression

$$v_i/v_0 = \frac{1}{1 + I/K_a'(1 + K_a'/H)},$$

in which K_a' = approx. 10^{-10}, fits the experimental curve for inhibition of APME



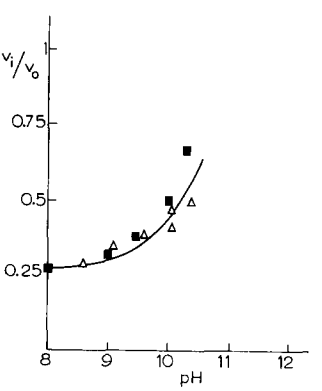


Fig. 7. Effect of pH on trypsin-catalyzed hydrolysis of APME (concn., $2 \cdot 10^{-3}$ M), in absence (×) and in presence (●) of $5.6 \cdot 10^{-5}$ M benzamidine. Initial rate v is in arbitrary units; cell volume, 10 ml; θ, 15°; ionic strength, 0.1; [enzyme], 0.18 mg/ml = $7.7 \cdot 10^{-6}$ M; potentiometric measurements.

Fig. 8. Effect of pH on the inhibition of trypsin-catalyzed hydrolysis of APME by benzamidine (△) and BA (■) (ref. 1); [APME], $2 \cdot 10^{-3}$ M; [benzamidine], $5.6 \cdot 10^{-5}$ M; [BA], 10^{-2} M. The inhibition is expressed by the ratio of the hydrolysis velocities in presence and in absence of inhibitor. The curve is calculated for a K_a' value of 10^{-10}.

TABLE II

INHIBITION OF BAEE TRYPTIC HYDROLYSIS BY BENZAMIDINE

Spectrophotometric data: $(\varepsilon_{BA} - \varepsilon_{BAEE})_{251\ m\mu} = 10^3$; [BAEE], 10^{-4} M; [benzamidine], $5.6 \cdot 10^{-4}$ M; [trypsin], $3.5 \cdot 10^{-8}$ M; θ, 15°; ionic strength, 0.3.

pH	v_i/v_o
8.60	0.41
10.40	0.51
10.85	0.52
11.00	0.44

hydrolysis by benzamidine or *N*-α-benzoyl-L-arginine (Fig. 8). The affinity of these inhibitors for trypsin decreases in the alkaline pH range, following the ionization of one enzymic residue of pK = 10. This group is without doubt the one responsible for the decrease in the esterasic activity (as measured with APME) and for the conformational change in the free enzyme; that is to say $K_a' = K_a = K_{II}$ (see Scheme 1).

(*b*) The tryptic hydrolysis of a specific substrate, BAEE, in the presence and absence of benzamidine, has been measured by a spectrophotometric method between pH 8.6 and 11. The experimental values of the rate ratio v_i/v_0 are given in Table II. The ratio v_i/v_0 remains approximately constant at any pH. The same result was obtained for inhibition by butylamine[1]. This finding appears to contradict the above observation concerning APME hydrolysis. However, the apparent contradiction may be reconciled by a careful investigation of the kinetic constants K_m and V for tryptic hydrolysis of BAEE in this alkaline pH range.

It has been previously shown that the rate of the BAEE tryptic hydrolysis, at concentrations from 10^{-4} to 10^{-3} M is constant between pH 8 and 11.5, and it was assumed that the kinetic constants K_m and V did not change with pH [22]. In fact, at a very low concentration of substrate ($5 \cdot 10^{-5}$ M), a clear decrease of the hydrolysis velocity is observed when the pH increases (Fig. 9): the order of the reaction is zero

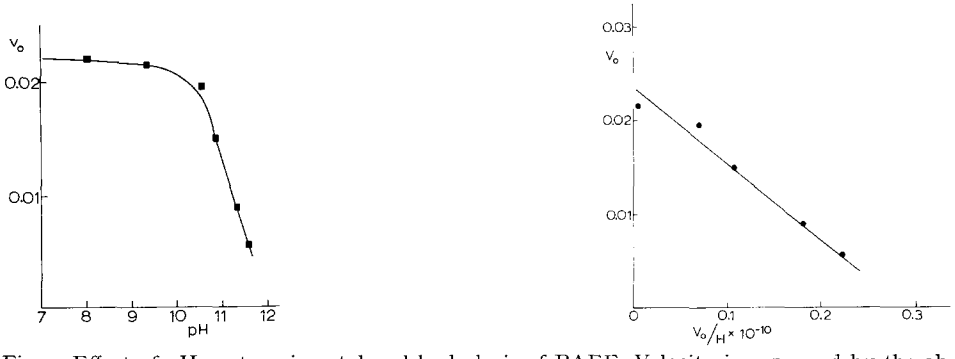


Fig. 9. Effect of pH on trypsin-catalyzed hydrolysis of BAEE. Velocity is expressed by the absorbance increase at 251 mμ as a function of time: ΔA = 0.01 corresponds to the hydrolysis of 10^{-5} M BAEE, $(\varepsilon_{BA} - \varepsilon_{BAEE})_{251\ m\mu} = 10^3$; cell volume, 3 ml; θ, 15°; ionic strength, 0.3; [enzyme], 0.82 μg/ml = $3.5 \cdot 10^{-8}$ M; [BAEE], $5 \cdot 10^{-5}$ M.

Fig. 10. Trypsin-catalyzed hydrolysis of BAEE: plot of velocity v_o *versus* v_o/H. Experimental conditions are the same as for Fig. 9.

at neutral pH and becomes unity at pH > 11. The kinetic constant K_m is then affected by the ionization of an enzymic group. One may write:

$$v_0 = \frac{V \cdot S}{S + K_m(1 + K_a/H)}$$

equivalent to

$$v_0 = \frac{V \cdot S}{K_m + S} - \frac{v_0 \cdot K_a}{H} \cdot \frac{K_m}{K_m + S}$$

The plot v_0 *versus* v_0/H is a straight line, whose slope is $-K_a\,(K_m/(K_m + S))$ (Fig. 10). The kinetic constant K_m being $4 \cdot 10^{-6}$ M at neutral pH, a pK_a of 9.95 is calculated. The same result was found in the tryptic hydrolysis of TAME[22].

This K_m increase with pH offers an explanation as to why the inhibitory power of benzamidine (and of butylamine) seems to be pH independent. Indeed, the reaction velocity in the presence of inhibitor is

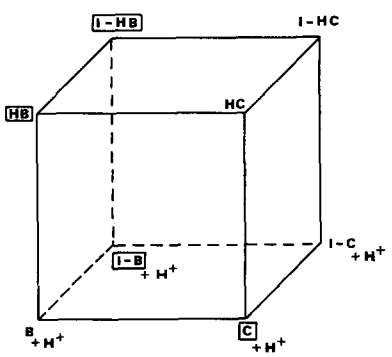
$$v_i = \frac{V \cdot S}{S + K_m(1 + K_a/H)(1 + I/K_{i(pH)})}$$

and the ratio v_i/v_0 is:

$$\frac{S + K_m(1 + K_a/H)}{S + K_m(1 + K_a/H)(1 + I/K_{i(pH)})}$$

Under the experimental conditions of this study ($[S] = 10^{-4}$ M; $[I] = 5.6 \cdot 10^{-4}$ M) and with the given values of $K_{m\,(pH\,8)} = 4 \cdot 10^{-6}$ M, $K_{i\,(pH\,8)} = 2.7 \cdot 10^{-5}$ M and $K_a = 10^{-10}$, the ratio v_i/v_0 will be 0.51 at pH 8 and 0.12 at pH 11, if the inhibition constant K_i is pH independent, while it will be equal to 0.51 at pH 8 and to 0.59 at pH 11, if the constant K_i increases following the ionization of an enzymic group having $pK = 10$. It is then clear that the inhibition of the tryptic hydrolysis of BAEE (or APME) by benzamidine, or by other inhibitors such as butylamine or BA, is pH dependent. In the alkaline pH range, an enzymic group of $pK = 10$ is responsible for the inhibitor's decrease in affinity.

All the results may be interpreted, to a first approximation, from the following



Scheme 3. Scheme showing equilibria between the different forms of trypsin and trypsin–inhibitor complex in the alkaline pH range. The main forms of free trypsin and complex in the pH range 8–11 are placed in squares. See explanations in text.

theoretical Scheme 3*, analogous to that presented by HIMOE *et al.*[23] and MCCONN *et al*[24] for chymotrypsin. In Scheme 3, it is assumed that the four forms of trypsin—HB, B, HC, and C—can bind the inhibitor. From the polarimetric results, it is evident that, in the pH range studied here (9–11.5), only the HB and B forms can bind the substrate, and then only the complexes *I*–B and *I*–HB are formed; so the equilibrium between HB and C is displaced in favor of HB (and B) and the apparent equilibrium constant is increased from 10 to over 11.5.

3. Experimental results in the acidic pH range

A previous study[1] of the interaction between trypsin and benzylamine in the acidic pH range has shown that: (a) The pH dependent rotatory power of the enzyme becomes pH independent when benzylamine is bound. (b) The fixation of inhibitor produces a proton release from the protein. An enzymic group having a pK of 4.7 is involved in this phenomenon; its protonation is responsible, at least partly, for the decrease in affinity of benzylamine for trypsin at pH < 5. This anionic group interacts with the positive charge of the substrate.

A new quantitative analysis of these phenomena has been made with a stronger inhibitor, benzamidine, and a scheme for the interaction is now presented.

Comparative rotatory power of trypsin and trypsin–benzamidine

In Fig. 2, the optical rotation $[\alpha]_{350\ m\mu}$ of the free enzyme, between pH 1.5 and 6.5 is compared with that of the benzamidine–trypsin complex. At any pH, the addition of excess benzamidine induces a lowering of the levorotation to a new value similar to that observed at neutral pH. According to Scheme 1, the equilibrium HAH $\rightleftharpoons$ HB + H^+ is displaced to the form HB. This change in rotatory power allows

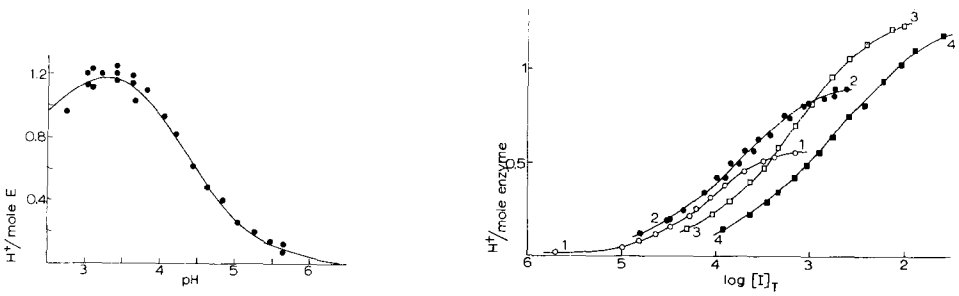


Fig. 11. pH dependence of the number of protons released by one enzyme molecule during trypsin–benzamidine interaction, in the acidic pH range; θ, 15°; ionic strength, 0.3; [enzyme], $8.9 \cdot 10^{-5}$ M; [benzamidine], $1.1 \cdot 10^{-2}$ M. 0.15 ml of a benzamidine solution (0.24 M) at a given pH was added to 3.1 ml of non-buffered trypsin solution at the same pH. The parameter H^+ released/mole of trypsin is calculated on the basis of 83% active sites (*p*-nitrophenylacetate titration). The curve is calculated (see text).

Fig. 12. Number of protons released by one molecule of trypsin as a function of benzamidine concentration during trypsin–benzamidine interaction; θ, 15°; ionic strength, 0.3. Curves: 1, pH 4.55; 2, pH 4.0; 3, pH 3.5; 4, pH 3.1. The parameter H^+ released/mole of enzyme is calculated on the basis of 83% active sites (*p*-nitrophenylacetate titration).

* In Scheme 3, no mention is made of the possible isomerization of trypsin after inhibitor binding, as observed at neutral pH with some inhibitors (1st part). Both phenomena were assumed to be independent in this discussion, although they may be related and explain some different kinetic results obtained with amides and native or chemically modified trypsin in the alkaline pH range[8].

TABLE III

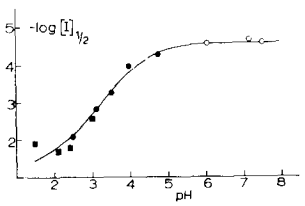
APPARENT DISSOCIATION CONSTANTS OF THE TRYPSIN–BENZAMIDINE COMPLEX, DETERMINED AT DIFFERENT pH'S BY 3 METHODS

Method	*pH*	$I_{1/2}$ *(M)*
Inhibition kinetic measurements	7.5	$2.6 \cdot 10^{-5}$
	7.0	$2.6 \cdot 10^{-5}$
	6.0	$3.25 \cdot 10^{-5}$
Potentiometric titration	4.55	$5.25 \cdot 10^{-5}$
	4.0	$8.9 \cdot 10^{-5}$–$1.3 \cdot 10^{-4}$
	3.5	$5.6 \cdot 10^{-4}$
	3.1	$1.6 \cdot 10^{-3}$
Polarimetric titration	2.95	$2.6 \cdot 10^{-3}$
Potentiometric titration	2.5	$1.0 \cdot 10^{-2}$
Polarimetric titration	2.4	$1.6 \cdot 10^{-2}$
	2.1	$1.9 \cdot 10^{-2}$
	1.55	$1.3 \cdot 10^{-2}$

one to determine the apparent dissociation constant $I_{\frac{1}{2}}$ of the trypsin–benzamidine complex at different pH's between 3 and 1.5. $I_{\frac{1}{2}}$ is evaluated from the plot log $([\alpha]_E - [\alpha])/([\alpha] - [\alpha]_{EI})$ *versus* log $[I]_T$. The concentration $[I]_T$ is the total inhibitor concentration; $[\alpha]$ is the rotatory power of trypsin for a given inhibitor concentration; $[\alpha]_E$ and $[\alpha]_{EI}$ are, respectively, the rotatory powers of the free enzyme for $[I] = 0$ and of the complex for $[I] = \infty$. The different values of $I_{\frac{1}{2}}$ obtained by this method are given in Table 3.

Proton release in the interaction of trypsin and benzamidine between pH 3 and 5.5

According to the preceding results (and also to the results obtained in the alkaline pH range), the apparent pK of the group which causes the conformational change in the free enzyme, the value of which is 3.7, must be shifted in the complex to a lower value. Therefore the trypsin–inhibitor association at a given pH will be accompanied by a proton release due to this pK difference. Moreover, proton release may also be due to the pK lowering of the anionic group of the binding site having a normal pK of 4.7.



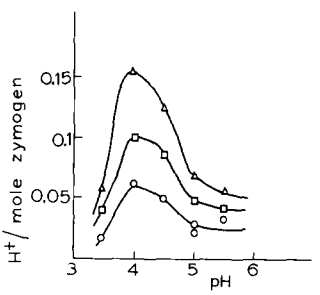


Fig. 13. pH dependence of the apparent dissociation constants of trypsin–benzamidine complex $I_{1/2}$ values were determined either by tryptic hydrolysis inhibition (○), by potentiometric titration (●) or by polarimetric titration (■).

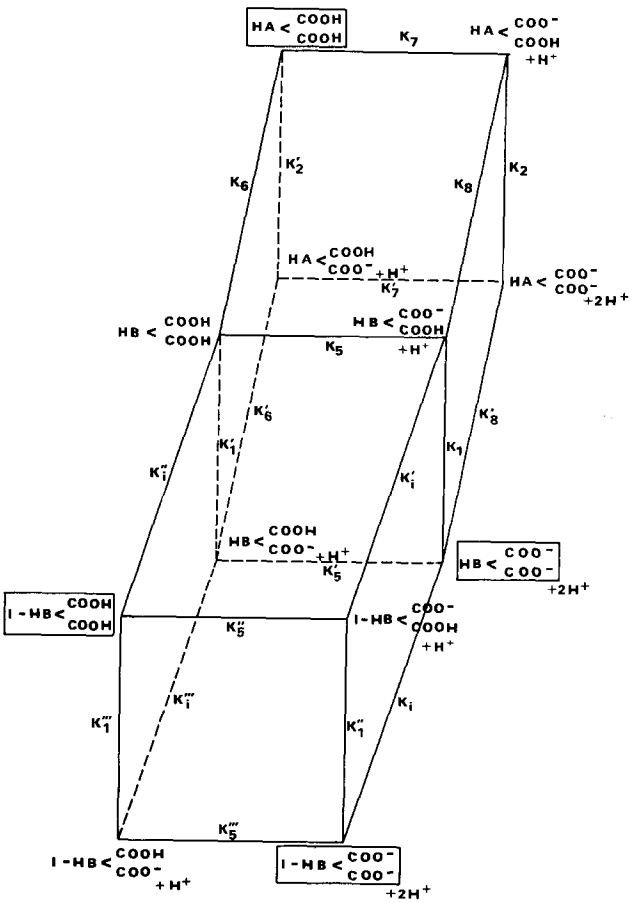
Fig. 14. pH dependence of the number of protons released by one molecule of trypsinogen during trypsinogen–benzamidine interaction; θ, 15°; ionic strength, 0.3; [trypsinogen], 3 mg/ml = $1.25 \cdot 10^{-4}$ M; final benzamidine concn., $4.2 \cdot 10^{-2}$ M (△); $3.2 \cdot 10^{-2}$ M (□), and $1.8 \cdot 10^{-2}$ M (○).

The number of instantaneously released protons by one molecule of trypsin in the presence of excess benzamidine is given in Fig. 11. The maximal value is greater than unity; the pK's of at least two anionic groups are shifted in the complex. The titration of released protons as a function of inhibitor concentration has been made at different pH's, between 2.5 and 4.5 (Fig. 12). The dissociation constants $I_{\frac{1}{2}}$ are calculated from the expression:

$$\log\left(\frac{H^+_{lim}}{H^+} - 1\right) = \log I_{1/2} - \log\left(I_T - \frac{H^+}{H^+_{lim}} E_T\right)$$

in which H^+ is the number of protons released by one molecule of trypsin, for a given inhibitor concentration, and H^+_{lim} the maximal number of protons released by the enzyme in the presence of excess inhibitor. The values of $I_{\frac{1}{2}}$ obtained by this method are given in Table III, and the pH dependence of $I_{\frac{1}{2}}$ is represented in Fig. 13.

This proton release is specific for trypsin. The addition of benzamidine to



Scheme 4. Scheme showing equilibria between different forms of trypsin and trypsin–inhibitor complex in the acidic pH range. The main forms of trypsin and complex are placed in squares. See explanations in text.

trypsinogen induces, between pH 5.6 and 3.5, a negligible pH-dependent proton release, only observable at very high benzamidine concentrations (Fig. 14). The dissociation constant of the benzamidine–trypsinogen complex is estimated to be approx. $5 \cdot 10^{-2}$ M at pH 5.6 instead of $2.7 \cdot 10^{-5}$ M found for the trypsin–benzamidine complex.

Scheme for the interaction of trypsin and benzamidine in the acidic pH range

The binding of benzamidine to the different neutral and acidic forms of trypsin (left side of Scheme 1) may be represented as in Scheme 4. In this scheme, the ionization states of both carboxylic groups, in the "B" and "A" enzymic forms, are indicated in the following way: in the symbol HA(or HB)$\begin{matrix}\text{COOH}\\ \text{COOH}\end{matrix}$, the upper carboxyl designates the carboxyl group responsible for the conformational change, and the lower one, the substrate binding carboxylic group. The main forms of the free enzyme are, respectively HB$\begin{matrix}\text{COO}^-\\ \text{COO}^-\end{matrix}$ at neutral pH, and HA$\begin{matrix}\text{COOH}\\ \text{COOH}\end{matrix}$ at very low pH. These forms are in equilibrium with other intermediate forms, depending particularly on the ionization states of both carboxylic groups. The different equilibrium constants are indicated in the scheme.

Theoretically, it may be assumed that, depending on the pH value, the inhibitor may be bound to the different enzymic forms. In fact, polarimetric measurements indicate that only the "B" forms of trypsin can bind benzamidine: the rotatory power of the complex is pH independent while its dissociation constant approaches a limit at low pH. The main molecular forms of the complex are then respectively I–HB$\begin{matrix}\text{COO}^-\\ \text{COO}^-\end{matrix}$ at neutral pH, and I–HB$\begin{matrix}\text{COOH}\\ \text{COOH}\end{matrix}$ at very low pH.

From this scheme, some pH dependent parameters may be calculated such as the apparent dissociation constant of the inhibitor–enzyme complex $I_{\frac{1}{2}}$, and the number of protons released by one molecule of trypsin in presence of excess inhibitor H^+/E_T. Several reasonable assumptions have been made for the values of the different constants of the scheme:

(a) The ionizations of both carboxylic groups in the free enzyme or complex are assumed to be independent of each other: $K_5 = K_5'$, $K_1 = K_1'$, $K_7 = K_7'$, $K_2 = K_2'$, $K_5'' = K_5'''$, $K_1'' = K_1'''$.

(b) The ionizations of the binding site carboxylic group in the "B" and "A" forms are assumed to be identical: $K_1 = K_2$ and consequently $K_8 = K_8'$. $K_1' = K_2'$ and consequently $K_6 = K_6'$.

(c) The inhibitor's affinity for the "B" forms depends only on the ionization state of the binding site carboxylic group, and not on that of the carboxylic group responsible for the enzymic conformational change. (This assumption is not strictly necessary for the calculations, but it seems logical according to the analysis of experimental data.) $K_i = K_i'''$ and consequently $K_5' = K_5'''$. $K_i' = K_i''$ and consequently $K_5 = K_5''$. These different relationships lead to the following equalities: $K_5 = K_5' = K_5'' = K_5'''$. $K_1 = K_1' = K_2 = K_2'$.

The apparent dissociation constant of the inhibitor–enzyme complex is the inhibitor concentration for which Σ (different forms of the free enzyme) is equal to Σ (different forms of the complex); it is given by the expression:

$$I_{1/2} = K_i \frac{\left(1 + \frac{1}{K_8}\right) + \frac{H}{K_5}\left(1 + \frac{1}{K_6}\right) + \frac{H}{K_1}\left(1 + \frac{1}{K_8}\right) + \frac{H^2}{K_1}\left(\frac{1}{K_5} + \frac{1}{K_5 K_6}\right)}{1 + \left(\frac{H}{K_1''} + \frac{H}{K_5'''}\right) + \frac{H^2}{K_1'' K_5''}}$$

Similarly, the number of protons released by one molecule of trypsin in the presence of excess inhibitor is:

$$H^+/E_T = \frac{1}{1 + H/K_1''} - \frac{1}{1 + H/K_1} + \frac{1}{1 + H/K_5'''} - \frac{1}{1 + \frac{H}{K_5 K_6} \cdot \frac{1 + K_6}{1 + 1/K_8}}$$

in which the last term is not very different from $1/1 + (H/K_5 K_6)$.

The theoretical curves of H^+/E_T and $I_{\frac{1}{2}}$ *versus* pH, which give the best fit to the experimental ones (Figs. 11 and 13) have been calculated using the following values: the dissociation constant K_i obtained by kinetic measurements is $2.7 \cdot 10^{-5}$ M; the ionization constant K_1 evaluated from potentiometric titration is approx. $3.2 \cdot 10^{-5}$; and the relation $K_1 = K_5 K_6 = K_7 K_8 = 2 \cdot 10^{-4}$, leads to the inequalities $K_6 < 1$, $K_5 > 2 \cdot 10^{-4}$ and $K_8 > 1$, $K_7 < 2 \cdot 10^{-4}$. Indeed, the assumption of a pH dependent equilibrium between two conformations of the enzyme, with conformation "B" predominant at neutral pH and conformation "A" predominant in the acidic pH range, requires $K_6 < 1$ and $K_8 > 1$ (for discussion see refs. 23, 24). The calculations have been made using the following plausible values: $K_7 = 10^{-5}$ (this is the value of the dissociation constant of a normal carboxylic group), $K_8 = 2 \cdot 10^1$, $K_5 = 2 \cdot 10^{-2}$, $K_6 = 10^{-2}$; and the K_i', K_1'' and K_5''' values which give the best agreement with the experimental results are, respectively, $5.4 \cdot 10^{-4}$, $6.3 \cdot 10^{-4}$ and $2 \cdot 10^{-2}$ M.

Thus, the binding of benzamidine to trypsin induces the lowering of the ionization pK of the substrate-binding carboxylic group from 4.5 ($K_1 = 3.2 \cdot 10^{-5}$) to the new value 3.2 ($K_1'' = 6.3 \cdot 10^{-4}$). Moreover the apparent pK_I (equal to pK_5 + pK_6) of the carboxylic group responsible for the enzymic conformational change seems to be lowered in the complex from 3.7 to 1.7. In fact, this last pK is a true pK (equal to pK_5'''), characteristic of a simple equilibrium.

These pK changes explain the pattern of the curve $I_{\frac{1}{2}}$ *versus* pH. The value of $I_{\frac{1}{2}}$ increases from $2.7 \cdot 10^{-5}$ M when carboxylic groups are ionized, to $3.1 \cdot 10^{-2}$ M when they are protonated. As the rotatory power of the trypsin–inhibitor complex is constant in the entire pH range, the protection by the substrate against the acidic denaturation is complete. At very low pH, the inhibitor is bound only to the native forms "B" and not at all to the denatured forms "A". This result suggests that, in the alkaline pH range, the forms HC and C may not bind the substrate and that only the forms I–HB and I–B exist in equilibrium with an apparent ionization constant higher than 11.5. A similar interpretation has been given by GAREL *et al.*[26], for acetylated δ-chymotrypsin.

CONCLUSION

The pH dependent binding of benzamidine and of other competitive synthetic inhibitors to trypsin has been studied between pH 1.5 and 11.5.

In the pH range from 5 to 8.5, where the preponderant form of trypsin is HB, the

formation of the enzyme–inhibitor complex induces a change in the optical properties of both the inhibitor and the protein (the larger the apparent affinity of the inhibitor, the more important is the observed effect). However, for the trypsin–butylamine complex, it has been shown that the complex formation induces no significant change in the enzyme structure: at neutral pH the trypsin molecule has a conformation and an ionization state favorable to the binding of an inhibitor.

In contrast, the results obtained at pH < 5 and > 8.5 indicate that the "A" and "C" forms of trypsin do not bind the inhibitor; the complex formation in the acidic and alkaline pH ranges induces an equilibrium shift in favor of the "B" forms. Indeed, it has been shown that the affinity of the inhibitor for trypsin decreases following the apparent ionization constant of the two groups of apparent pK 3.7 and 10.1 which control the conformational change of the free enzyme. In the acidic pH range, moreover, there is an affinity decrease due to the protonation of the carboxylic group of the enzyme binding site (p$K_1 = 4.5$).

In conclusion, it can be said that the formation of the trypsin–inhibitor complex maintains the "B" forms of the protein over a large pH range (1.5 to 11.5) and therefore protects the enzyme against acidic and alkaline reversible denaturations.

ACKNOWLEDGMENTS

This work was supported by grants from the "Centre National de la Recherche Scientifique" (E.R. 19 and R.C.P. 23) and the D.G.R.S.T. (Biologie Moléculaire, conventions No. 6700603 and No. 6700580).

We are grateful to Prof. J. Tonnelat, Dr. J. Yon and Dr. S. Guinand for their interest in this work and for many helpful discussions. We also thank Dr. Thusius for carefully reading this manuscript.

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