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BENZOYLAMIDOACETONITRILE AS AN INHIBITOR OF PAPAIN

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SUMMARY

1. With benzoylarginine ethyl ester as a substrate, benzoylamidoacetonitrile is a strong competitive inhibitor of papain (EC 3.4.4.10) (K_i = 0.14 mM).

2. The inhibitor is not a poor substrate.

3. The binding of the inhibitor is governed by groups of pK 3.7 and 8.5, whereas the overall activity (k_{cat}/K_m) is governed by groups of pK 4.2 and 8.5.

4. In the enzyme–inhibitor complex the thiol group is completely protected against alkylation by chloroacetic acid.

5. The possibility that the inhibitor is a transition state analog is discussed.

INTRODUCTION

Lucas and Williams[1] have reported that benzoylamidoacetonitrile (BAAN), which closely resembles the structure of the substrate benzoyl glycine amide, is an excellent competitive inhibitor of papain (EC 3.4.4.10). Furthermore they have reported that the extent of inhibition is independent of the pH in the range of pH 4.0 to 8.5. Most striking is the observation that the binding of the substrate substitute (K_i = 0.4 mM) is considerably stronger than the binding of the substrate proper (K_m = 0.16 M).

Therefore it seemed worthwhile to re-examine and extend the data of Lucas and Williams[1]. The results, which differ in some respects from the results of these authors, are presented in this paper.

EXPERIMENTAL

Mercuric papain was prepared by elution from an agarose mercurial column[2]. Benzoylarginine ethyl ester (BAEE) was purchased from Fluka (Switzerland), benzoyl-DL-arginine *p*-nitroanilide (BAPA) from Merck (Darmstadt) and dithiothreitol from Calbiochem (Luzern). Acetylglycine ethyl ester (AGEE) was prepared

Abbreviations: BAAN, benzoylamidoacetonitrile; BAEE, benzoylarginine ethyl ester; AGEE, acetyl glycine ethyl ester; BAPA, benzoyl-DL-arginine *p*-nitroanilide.

according to the method of Curtius and Goebel[3] and BAAN by that of Klages and Haack[4].

Rates of hydrolysis of AGEE and BAEE were measured in the pH-stat of Radiometer (Copenhagen) composed of titrator TTT1, titrigraph SBR2, syringe burette SBU1 and electrode head Type D 4511. Since the all-glass syringe occasionally gets stuck, a "pressure lok" 0.5-ml syringe ("Precision Sampling Corp.", Louisiana) was used, which delivered 0.2 ml 0.05–0.1 M NaOH at maximum deflection of the recorder. A salt bridge containing 2% agar and 1 M KCl was inserted between reaction mixture and calomel electrode in order to prevent dithiothreitol from entering and affecting the electrode. Water at 25 °C was circulated through the water jacket of the reaction vessel. The progress curves were sufficiently straight at all pH values to allow a straightforward determination of initial rates and no extrapolation to zero time[1] was required. The rates in the acid range were corrected for incomplete ionization of the product, assuming pK 3.67 for acetylglycine[5] and pK 3.38 for benzoylarginine[6]. The rate of hydrolysis of BAPA was determined in a spectrophotometer Unicam SP1800 at 420 nm, and cells of 2-cm optical pathway inserted in thermospacers of 25 °C. The 10-ml reaction mixture in the vessel of the pH-stat usually contained 0.3 M KCl, 1 mM EDTA and 2.5 mM dithiothreitol in the pH range 3.7 to 8.0 and 0.5 mM dithiothreitol at pH $>$ 8.0. This low concentration of activator at the higher pH values proved to be sufficient for complete activation and did not interfere with the proper action of the pH-stat as did 2.5 mM activator (owing to its buffering action at pH around 9). In the cell of the spectrophotometer the medium was the same and 2.5 mM dithiothreitol was used throughout.

For the determination of overall activity (Fig. 2) the substrate concentrations were taken at least five times lower than K_m, *i.e.* BAEE was 3.5 mM (K_m = 17 mM), AGEE 20 mM (K_m = 0.45 M, ref. 7), and BAPA 0.15 mM (K_m = 2.8 mM, ref. 8). At the extreme values of pH, where K_m values increase, the concentrations were doubled and the observed rates were divided by two in order to make them comparable with the rates in the neutral range.

The inhibitor was added dissolved in acetonitrile. The final reaction mixture contained no more than 1% of acetonitrile, an amount which was found not to affect papain activity.

In order to test whether BAAN is consumed by papain, a solution of 10% acetonitrile, 0.05 M acetate (pH 6.0), 5 mM BAAN, 1 mM EDTA, 2.5 mM activator and $1 \cdot 10^{-5}$ M papain was stored for 2.5 h at 25 °C. The solution was acidified to pH 2 and stored for 15 min at room temperature to ensure inactivation of the enzyme. Aliquots of the resulting solution were used for testing its inhibitory action and compared with control solutions in which papain or the inhibitor had been omitted. The preincubation with papain appeared not to affect the BAAN concentration.

RESULTS

Type of inhibition

At three pH values the inhibition of the papain-catalyzed hydrolysis of BAEE by BAAN was investigated at a single concentration of BAAN and several concentrations of substrate.

The data were plotted according to the method of Lineweaver and Burk and

TABLE I

INHIBITION OF THE PAPAIN-CATALYZED HYDROLYSIS OF BAEE BY BAAN

pH	*[BAEE] (mM)*	*[BAAN] (mM)*	*No. of experimental points*	*Rel. V*	K_m *(mM)*	K_i *(mM)*
3.8	10–40	0	7	1.00 ± 0.06	62 ± 5	
						0.24 ± 0.01
3.8	10–40	0.364	7	1.00 ± 0.10	158 ± 17	
6.0	10–40	0	8	1.00 ± 0.02	17 ± 0.5	
						0.13 ± 0.01
6.0	10–40	0.302	8	1.03 ± 0.07	57 ± 6	
8.6	10–40	0	8	1.00 ± 0.07	22 ± 3	
						0.30 ± 0.05
8.6	10–40	0.364	8	0.95 ± 0.12	46 ± 8	
3.8	10 and 50	0.25–1.0	6	—	—	0.23 ± 0.01
6.0	10 and 50	0.25–1.0	6	—	—	0.15 ± 0.01
8.4	10 and 50	0.25–1.0	6	—	—	0.27 ± 0.02

regression lines were determined in a computer by the method of least squares, assuming an equal relative error in each experimental point. Within experimental error the maximum velocities were equal in the presence and in the absence of the inhibitor (Table I), indicating competitive inhibition. This is a qualitative confirmation of the results of Lucas and Williams[1]. However, the present value of K_i at pH 6 is 0.13 mM, *i.e.* lower than their value of 0.4 mM at 35 °C. Furthermore the data of Table I indicate that the inhibition is strongly pH-dependent.

The inhibition was also examined at two different substrate concentrations and several BAAN concentrations. The data were plotted as $1/v$ *versus* inhibitor concentration, after Dixon, and regression lines determined. Again competitive and pH-dependent inhibition was observed (Table I).

pH dependence of the inhibition

In order to compare the pH dependence of the inhibition with the pH dependence of the overall activity, the activity of papain was determined as a function of pH in the absence and in the presence of a single inhibitor concentration and of a single low substrate concentration $[S]$. When $[S] \ll K_m$ the dissociation constant of the EI complex is equal to

$$K_i = i/\left(\frac{v}{v_0} - 1\right) \qquad (1)$$

In Fig. 1 the inhibition is plotted as $1/K_i$. Overall activity and inhibition are plotted as a percentage of maximum values. In all three cases bell-shaped curves were obtained; the basic branches of the curves of activity and of inhibition coincide (pK 8.5), whereas the acid branches of the inhibition proved to be located at a pH value of 0.5 unit lower (pK 3.7) than the acid branch of the activity curve (pK 4.2).

Blockage of the thiol group

The reactivity of the thiol group of papain towards chloroacetic acid in the presence of various concentrations of the inhibitor was investigated by a method

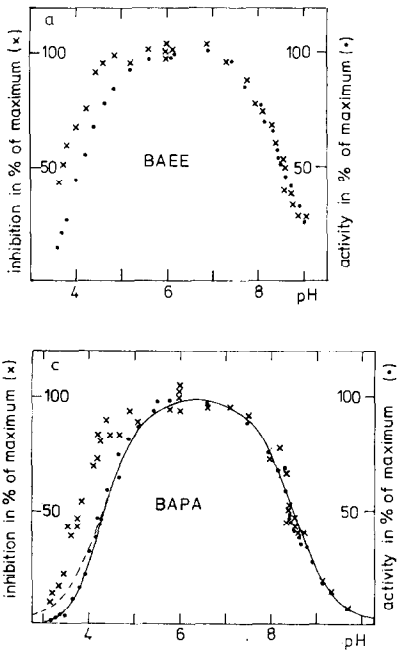

Fig. 1. Overall activity and inhibition of papain towards three substrates as a function of pH. (a) BAEE. (b) AGEE. (c) BAPA. Drawn curves: single ionization of pK 8.5 (right-hand branch) and double ionization of two groups of pK 3.95 (left-hand branch). Broken curve, single ionization of pK 4.25.

described previously[9]. In the reaction vessel of the pH-stat, solutions of substrate, inhibitor and active papain are mixed. After the reaction has progressed for a few minutes a small aliquot of neutralized chloroacetic acid is added. The papain is gradually inactivated by the alkylation of the thiol group. Consequently the progress curve levels off until the reaction stops completely. From the shape of this part of the curve the rate of alkylation can be derived[7]. The latter rate can be determined as a function of inhibitor concentration. Since the total enzyme concentration, e, is distributed as concentration of free enzyme, $[E]$, enzyme substrate complex, $[ES]$, and enzyme inhibitor complex, $[EI]$, the apparent rate of alkylatin k_a, equals:

$$k_a e = k_f [E] + k_s [ES] + k_i [EI] \tag{2}$$

where k_f, k_s and k_i are rates of alkylation of free enzyme, of ES and of EI complexes, respectively. The substrate employed was 20 mM BAEE. For this substrate it has previously been found that $k_s = k_f$ (ref. 7). Hence:

$$k_a e = k_f([E] + [ES]) + k_i[EI] \tag{3}$$

Application of conventional Michaelis–Menten treatment yields:

$$k_a = \frac{k_f \left(1 + \frac{[S]}{K_m}\right) + k_i \frac{[I]}{K_i}}{\left(1 + \frac{[S]}{K_m} + \frac{[I]}{K_i}\right)} \tag{4}$$

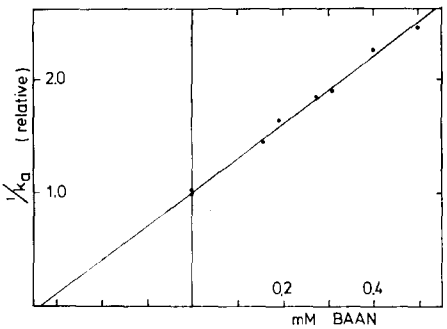

Fig. 2. Rate of inactivation of papain by chloroacetic acid (1.5 mM) as a function of BAAN concentration in the presence of BAEE (21 mM) at pH 6.0, 25 °C.

If the thiol group is completely protected in the EI complex, $k_i = 0$ and Eqn 4 can be turned into:

$$\frac{1}{k_a} = \frac{1}{k_f} + \frac{[I]}{k_f K_i \left(1 + \frac{[S]}{K_m}\right)} \tag{5}$$

A plot of $1/k_a$ *versus* $[I]$ indeed resulted in a straight line (Fig. 2), indicating complete protection of the thiol group in the EI complex. Furthermore the intercept of the abscissa equals:

$$[I] = -K_i \left(1 + \frac{[S]}{K_m}\right)$$

Insertion of $[S] = 20$ mM and of $K_m = 17$ mM (Table I) yielded $K_i = 0.15$ mM, which is virtually equal to the values found above.

These data indicate complete protection of the thiol group in the enzyme inhibitor complex.

Is BAAN a substrate?

Its apparently low K_i value might be due to the fact that BAAN is actually a poor substrate with a relatively high rate of acylation of the enzyme and a low rate of deacylation, according to the reactions:

$$\text{R–C}\equiv\text{N} + E\text{–SH} \overset{K_s}{\rightleftarrows} [\text{R–C}\equiv\text{N}\cdot E\text{–SH}] \xrightarrow{k_2} \text{R–C(=NH)–S–}E + \text{H}_2\text{O} \xrightarrow{k_3} \text{R–C(=O)–NH}_2 + E\text{–SH}$$

Such reactions would result in an apparent K_i equal to:

$$K_i = K_m = K_s \frac{k_3}{k_2}$$

This possibility was tested in the following manner. In order to estimate the lower limit of k_3, enough BAAN (4 mM) was added to a stock solution of active papain at pH 6 to give 96% inhibition. After a few minutes an aliquot of 0.1 ml was

injected into the 10-ml reaction mixture in the vessel of the pH-stat containing all ingredients for assay (22 mM BAEE). The reaction was found to start instantaneously to its final extent. Even a time lag of 10 s to reach final activity would have been observed. This indicates that $k_3 \geqslant 0.1\ s^{-1}$. From this, calculation shows that incubation of $1 \cdot 10^{-5}$ M active papain with 5 mM BAAN (*i.e.* a concentration high enough to saturate all enzyme with inhibitor) for 2.5 h should reduce the concentration of BAAN considerably. Actually no decrease was observed, as established by the inhibitory action of the solution after removal of the enzyme. Hence BAAN is not a substrate.

DISCUSSION

Although the results of Lucas and Williams[1] and of the present authors agree in that BAAN is a strong inhibitor and a competitive one, the inhibitory action is now found to be stronger ($K_i = 0.14$ mM) than reported ($K_i = 0.4$ mM) and to be markedly pH dependent. No simple explanation of the discrepancy is evident, but the present results are based upon so many repeated experiments as to leave little doubt about their accuracy. The present data no longer provide a support for the argument that the binding of substrates is pH independent.

Binding evidently may be very sensitive to small changes in apparently essential parts of the structure of a ligand: binding of BAAN ($K_i = 0.14$ mM) to papain is three orders of magnitude stronger than the binding of the substrate benzoylglycine amide ($K_m = 0.16$ M, ref. 10). Binding is reduced even more if the $-C \equiv N$ group is replaced by the group $-CH_2OH$; *i.e.* benzoylamidoethanol is hardly an inhibitor at all ($K_i > 1$ M, ref. 1).

The great difference in binding of BAAN and of its related substrate suggests that the structure of BAAN may have something in common with the transition state[11]. Since the mechanism of papain action involves the acyl enzyme as an intermediate in which the acyl group is attached to the thiol group, the latter is no doubt involved in the transition state. If the enzyme–BAAN complex is like the transition state, one would expect the thiol group to be involved in the complex. This in fact is apparent both from the pH dependence in the basic branch of the binding (since pK 8.5 equals the pK of the thiol group, refs 12 and 13) and from the complete protection of the thiol group against alkylation in the complex.

If the interpretation of BAAN as a transition state analog is correct, the transition state is not tetrahedral and is strongly polarized.

As far as the acid branches of the curves of binding of BAAN and of overall activity are concerned, both are steeper than a simple titration curve, as is especially evident in the experiments with BAPA (Fig. 1c). This steepness could be caused by the simultaneous ionization of two groups of about equal pK (Fig. 1c, drawn curve). It could also be due to the salt effect previously reported[14]; decreasing pH increases the overall positive charge of the enzyme, and therefore increases the binding of anions and the apparent pK, resulting in an increasing shift of the curve towards the right as the pH decreases. This yields a steeper curve than the conventional one.

Whatever the explanation of the steepness may be, the binding of BAAN is governed by a group or groups with a pK 0.5 unit lower than that of the overall activity. It could be due to different groups being involved, or in some way to a shift

in pK of the same groups. It is premature to discuss this matter before the problem of the steepness is solved.

REFERENCES

1 Lucas, E. C. and Williams, A. (1969) *Biochemistry* 8, 5125–5135
2 Sluyterman, L. A. Æ. and Wijdenes, J. (1970) *Biochim. Biophys. Acta* 200, 593–595
3 Curtius, T. and Goebel, F. (1888) *J. Prakt. Chem.* 37, 150–154
4 Klages, A. and Haack, O. (1903) *Ber. Deut. Chem. Ges.* 36, 1646–1649
5 King, E. J. and King, G. W. (1956) *J. Am. Chem. Soc.* 78, 1089–1099
6 Sluyterman, L. A. Æ. (1964) *Biochim. Biophys. Acta* 85, 305–315
7 Sluyterman, L. A. Æ. and de Graaf, M. J. M. (1969) *Biochim. Biophys. Acta* 171, 277–284
8 Tokura, S., Nishi, N. and Noguchi, J. (1971) *J. Biochem. Tokyo* 69, 599–600
9 Sluyterman, L. A. Æ. (1968) *Biochim. Biophys. Acta* 151, 178–187
10 Smith, E. L., Chavré, V. J. and Parker, M. J. (1958) *J. Biol. Chem.* 230, 283–293
11 Collins, K. D. and Stark, G. R. (1971) *J. Biol. Chem.* 246, 6599–6605
12 Sluyterman, L. A. Æ. and de Graaf, M. J. M. (1970) *Biochim. Biophys. Acta* 200, 595–597
13 Chaiken, I. M. and Smith, E. L. (1969) *J. Biol. Chem.* 244, 5087–5094
14 Sluyterman, L. A. Æ. and de Graaf, M. J. M. (1972) *Biochim. Biophys. Acta* 258, 554–561