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BINDING OF COMPETITIVE (PRODUCT) INHIBITORS TO PAPAIN IN THE ACTIVE AND INACTIVE STATE

B. G. WOLTHERS AND K. H. KALK
Laboratory of Structural Chemistry, Bloemsingel 10, Groningen (The Netherlands)

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SUMMARY

1. In X-ray studies of crystalline papain product complexes, it was found that the active site was never occupied more than 30% with the product. This could be due to blocking of the essential SH in a fraction of the papain molecules while assuming that these non-active enzyme molecules do not bind the product. To prove this hypothesis product–inhibitor experiments were carried out in solution with Boc–*p*-I–Phe–Leu ($K_i \approx 1 \cdot 10^{-6}$ M at pH 4) and benzoylarginine, with $K_i \approx 0.03$ M at pH 4.

2. Two experimental approaches were applied. The first approach is applicable, when K_i, the dissociation constant of enzyme–product complex, has the same order of magnitude as the free enzyme concentration. In that case the fractional inhibition caused by the product depends on the enzyme concentration. In the second method the alkylation of papain by iodoacetic acid or iodoacetamide is measured as a function of the Boc–*p*-I–Phe–Leu or the benzoylarginine concentration.

3. The results obtained with both methods indicate that any blocking of the essential SH in papain prevents binding of products. The possibility is discussed that the observed binding constant K_i of a product must be ascribed to the formation of the corresponding acylenzyme.

INTRODUCTION

The crystal structure of papain has been determined by X-ray techniques[1]. With the knowledge of the active site structure and the structure of enzyme substrate or enzyme inhibitor complexes, one can hope to explain the mechanism of action of this enzyme.

Suitable competitive inhibitors (products) are available since SCHECHTER AND BERGER[2] found that peptides with a phenylalanyl residue in their sequence are split at the peptide bond one removed from the Phe residue towards the C terminal and also that the corresponding products are powerful competitive inhibitors. When the Phe ring at the *p*-position is substituted by an iodine atom, the binding constants

Abbreviations: BPL, Boc–*p*-I–Phe–Leu; BAEE, benzoylarginine ethyl ester.

improve even more. For instance, the compound Boc–*p*-I–Phe–Leu (BPL) has a binding constant (K_i) of about $1 \cdot 10^{-6}$ M, dependent on the pH.

Preliminary X-ray studies suggest that even with a high excess of inhibitor, the active site in the crystalline material was never occupied for more than 30% and usually less. Obviously in the crystals a major portion of the papain molecules can not bind the inhibitor. This low occupancy is a serious problem in high resolution X-ray work. Therefore the binding properties of these product inhibitors *viz.* benzoyl-arginine and BPL were investigated systematically.

Papain, prepared by the standard method of KIMMEL AND SMITH[3] usually is hardly active because of partial blocking of the essential free sulfhydryl group (Cys 25). By addition of cysteine and EDTA, the blocking groups can be removed from the SH group and papain then attains maximum activity[4]. This particular reaction is reversible. However it appears (after checking the free sulfhydryl content of activated papain preparations), that the expected value of 1 mole SH/1 mole of papain is never reached[5]. A certain fraction of a papain sample is irreversibly blocked. The nature of this blocking is still unknown but could probably be a higher oxidation state of the SH group, as suggested by GLAZER AND SMITH[6].

Summarizing: any papain solution can contain three kinds of molecules (1) active, (2) inactive, but activatable, (3) inactive and not activatable. In fully activated papain solutions only the first and third forms are present.

The purpose of this investigation is to find out which forms of papain bind the product. We were able to prove that only active papain can bind products. The significance of this result will be discussed in the last paragraph of this paper.

OUTLINE OF THE EXPERIMENTAL PROCEDURES

A number of approaches can be used to study the binding of products to the different forms of papain. First of all the extent of binding can be derived under conditions in which the concentration of enzyme and inhibitor are of the same order of magnitude (*Method 1*). Secondly the reaction of small sulfhydryl blocking reagents, *e.g.* the alkylating haloacetates, with enzyme–product complexes can be studied (*Method 2*). Finally in the third approach the product is added to the reversibly blocked enzyme. If subsequent activation is hampered by the product it is likely that the product was already bound to the blocked enzyme (*Method 3*). This last method was not used in the present investigation but a nice example of this approach is given in the work of SLUYTERMAN[7]. He showed that the activation of reversibly blocked papain was retarded in the presence of benzoylarginine ethyl ester (BAEE).

As the techniques used in this paper (*Method 1* and *Method 2*) are not very common, we shall give a short outline of our experimental procedures.

Method 1

The availability of very good competitive inhibitors suggested to us a useful method to establish which of the three forms of papain bind products.

By combining the Michaelis–Menten equation for the velocity v_0 of substrate conversion by an enzyme and the equation of the velocity v_i for the same substrate reaction in the presence of a competitive inhibitor one can obtain the following ex-

pression, giving the inhibitor concentration $[I]$, necessary for attaining a fractional inhibition i (equal to $(v_0-v_i)/v_0$):

$$[I] = \frac{i}{1-i} \cdot \frac{K_m+[S]}{K_m} \cdot K_i \tag{1}$$

K_m, Michaelis–Menten constant of the substrate; $[S]$, substrate concentration; K_i, binding constant of the inhibitor.

This formula is not quite correct, when for $[I]$ the total inhibitor concentration is taken. In fact, $[I]$ represents the free inhibitor concentration, that is the total concentration minus the fraction, bound by the protein. However, normally K_i is much larger than the enzyme concentration. In order to obtain a measurable degree of inhibition, the concentration of inhibitor has to be several times higher than the enzyme concentration. Consequently only a very small fraction of the inhibitor is taken away, leaving its free concentration practically unchanged. Therefore the degree of inhibition is nearly independent of the protein concentration.

When however K_i has the same order of magnitude as the enzyme concentration, the binding of the inhibitor to the enzyme then results in a considerably lower free inhibitor concentration as now the quantity of inhibitor, needed for an observable inhibition, is in the same range as the enzyme quantity. Consequently the degree of inhibition in this case is indeed dependent on the enzyme concentration, thereby making it possible to obtain information about the concentration of enzyme, capable of binding the inhibitor.

To derive quantitative formulae in the latter case, one has to deal with "inhibition in mutual depletion systems", as it is called by LEYDEN WEBB[8], who gives an extensive theoretical treatment based on the work of GOLDSTEIN[9].

The total inhibitor concentration $[I_t]\ (i)$, necessary for obtaining a fractional inhibition i, is written as:

$$[I_t](i) = [I_f](i) + [I_b](i) \tag{1a}$$

where $[I_f]$ and $[I_b]$ are, respectively, the free and bound inhibitor concentrations. For $[I_f]\ (i)$ Eqn. 1 holds, while $[I_b]\ (i)$ can be replaced by $[EI]$, the concentration of enzyme–inhibitor complex. We now can write:

$$[I_b]\ (i) = [EI] = \frac{[EI]}{[E_b]} \cdot [E_b] = i \cdot [E_b] \tag{2}$$

where $[E_b]$ is the enzyme concentration, capable of binding inhibitor and i is again the fractional inhibition. Combining Eqns. 1, 1a and 2 one obtains:

$$[I_t]\ (i) = \frac{i}{1-i} \cdot \frac{K_m+[S]}{K_m} \cdot K_i + i \cdot [E_b] \tag{3}$$

For 50% inhibition this reduces to:

$$[I_t]\ (0.5) = \frac{K_m+[S]}{K_m} \cdot K_i + 0.5\ [E_b] \tag{4}$$

If an enzyme preparation contains only a fraction f of enzyme capable of binding and

thus $[E_b] = f \cdot [E_t]$, in which $[E_t]$ denotes the total enzyme concentration, Eqn. 4 can be written as:

$$[I_t]\ (0.5) = \frac{K_m + [S]}{K_m} \cdot K_i + 0.5 f \cdot [E_t] \qquad (4a)$$

By determinating $[I_t]$ (0.5) for a number of different enzyme concentrations, $[E_b]$ can be calculated (see MATERIALS AND METHODS and RESULTS). For non-competitive inhibition the factor $(K_m + [S])/K_m$ disappears and the even simpler formula was used by MYERS[10] to determine the acetylcholine esterase concentration with a strong inhibitor.

Method 2

Method 1 is best applicable to inhibitors which have binding constants of the same order of magnitude as the enzyme concentrations, used in the inhibition experiments. *Method 2* does not have this limitation. A way to study the blocking of the active site was first given by SLUYTERMAN[11]. He determined the rate constant of the reaction of chloroacetic acid with papain in the presence of different concentrations of BAEE. The BAEE hydrolysis by papain was followed with a pH stat recorder. Then a certain amount of chloroacetic acid was added while the hydrolysis continued. Due to the reaction of chloroacetic acid with papain the hydrolysis rate diminished gradually. As the activity is directly proportional to the slope of the progress curve, the half time value of the inhibition reaction can be easily deduced from this curve (see Fig. 5). SLUYTERMAN[11] found that the reaction rate of chloroacetic acid was independent of the substrate concentration. This can only mean, that in the BAEE–papain complex the SH-group of Cys 25 is equally accessible as in the free enzyme. WOLTHERS[12] showed that even the reaction with the bulky tosyl-L-phenylalanine chloromethyl ketone molecule is not hindered in the BAEE–enzyme complex. The method lends itself also to the study of the behavior of competitive inhibitors. To the reaction mixture of BAEE and papain some inhibitor is added and next the alkylating reagent. If it turns out, that the alkylation rate is less than without the inhibitor, it proves that in the enzyme–product complex the free SH group will be partially or completely blocked, as the delay in alkylation can only mean, that the SH group in the enzyme–product complex is not easily accessible to the reagent.

MATERIALS AND METHODS

Materials

BPL was kindly provided to us by Dr. A. Berger. BAEE, KCl and iodoacetate all analytical grade, were from Merck, iodoacetamide (very pure) from Serva, cysteine, analytical grade from Noury Baker, EDTA from Siegfried AG.

Inhibition experiments were performed with a Radiometer Titrigraph TTT 1a, equipped with a SBU 1c buret and a SBR 2c recorder. All reactions were carried out in a 10-ml reaction vessel provided with a water jacket, thus making it possible to carry out the reaction at constant temperature by connecting the jacket to a thermostatically controlled water bath. BPL was dissolved as an approx. $1 \cdot 10^{-3}$ M aqueous solution and the exact concentration then was determined by acid–base titration, being $0.83 \cdot 10^{-3}$ M. This solution was diluted 5-fold and used throughout the experi-

ments. Papain concentrations were determined by a Zeiss-spectrophotometer. For $E\,{}^{1\%}_{1\,cm}$ at 280 nm a value of 25 as reported by GLAZER AND SMITH[13] was taken. The molecular weight of 23000 (ref. 1) was used. In one experiment papain crystals, grown from methanol–water (2:1, by vol.) crystal form C, were checked upon their activity. These two-year-old crystals were carefully selected, washed with methanol–water and finally dissolved in water.

Method 1

In many experiments fully (with EDTA + cysteine) activated papain was used. The reaction vessel then contained cysteine (5 mM), EDTA (1 mM), as substrate BAEE (5 mM) and 0.3 M KCl for maintaining a constant ionic strength. By addition of a papain solution the hydrolysis of BAEE was started and followed with a pH stat recorder at 25° and pH 4.0. The total volume was 10 ml and the solution was titrated with 0.0188 M NaOH. After a few minutes so much BPL was injected, that the hydrolysis rate was reduced to half its original value (50% inhibition) (Fig. 1). This was repeated for different concentrations of papain. In some cases only EDTA was used as an activator. In that case cysteine was absent from the reaction, described above.

Method 2

This method was applied both with BPL and benzoylarginine as inhibitors. The procedure outlined by SLUYTERMAN[11] was used. Instead of chloroacetic acid, iodoacetic acid or iodoacetamide were used as the alkylating agents. The procedure was followed for different BAEE concentrations and with or without additional amounts of BPL or benzoylarginine at pH 4.3 and 25°. When BPL was used as product, the papain concentration was much lower than the BPL concentration, in this way avoiding that during the alkylation reaction so much BPL would be released from the protein, that the free BPL concentration and therefore the inhibition would increase considerably.

Because of the rather low K_i value of benzoylarginine (at pH 4.3 about 0.03 M, as measured by SLUYTERMAN[14]), rather big quantities of benzoylarginine were required for a measurable inhibition. These high concentrations of benzoylarginine have a marked buffering effect, which makes the titration curves (titration with 0.0188 M NaOH) less accurate. However by using higher BAEE concentrations and 0.1 M NaOH as titrant these difficulties were overcome.

RESULTS

Method 1. Binding behavior of fully activated papain

Respectively, 0.4, 0.8, 1.2, 1.6 and 2.0 ml of a stock solution of freshly prepared papain (Sample I), E at 280 nm = 2.86, was added to reaction mixtures, described in the experimental procedure. In each hydrolysis reaction the amount of $1.66 \cdot 10^{-4}$ M BPL solution, needed for 50% inhibition, was determined. The results are given in Table I. The results in Table I are converted into molar concentrations of BPL and papain and plotted in Fig. 1.

From Fig. 1 it is easy to calculate the binding fraction of the papain solution using Eqn. 4a. The intercept $\{(K_m + [S])/Km\} \cdot K_i$ is observed to be $1.8 \cdot 10^{-6}$ M.

TABLE I

BPL SOLUTION (ml) NECESSARY FOR 50% INHIBITION OF VARIOUS AMOUNTS OF PAPAIN SOLUTION
Sample I, E at 280 nm = 2.86, final volume 10 ml.

Papain (ml)	0.4	0.8	1.2	1.6	2.0
BPL (ml)	0.15	0.19	0.23	0.26	0.30

From the slope a value of $f = 0.62$ is obtained, implying that 62% of the papain is capable of binding the inhibitor. The remaining 38% can not bind BPL and probably represents the irreversibly inactive fraction.

It is now possible to calculate roughly the activity corresponding to 100% active papain. In the experiments, just described, we experimentally observed that in the presence of 5 mM BAEE at pH 4 and 25° $1.0 \cdot 10^{-7}$ mole of added papain gave rise to a base consumption of $1.1 \cdot 10^{-7}$ mole NaOH per sec, *i.e.* 1.1. moles of NaOH per mole papain per sec. For 100% active papain this turnover rate should be $(100/62) \cdot 1.1 = 1.8$ moles NaOH per mole papain per sec. After corrections for non-ionized benzoylarginine at pH 4 (ref. 15) taking for K_m of BAEE at pH 4 a value of 0.04 M, k_0 of BAEE is calculated to be approx. 21 sec^{-1} for 100% active papain.

Some other papain preparations were checked upon their activity. The 3-month-old Sample II had a turnover, when fully activated, corresponding to an activity of 33%, and a 6-month-old sample (Sample III) was only 30% active. Finally we checked the activity of a papain solution, prepared from 2-year-old crystals (Sample IV). It

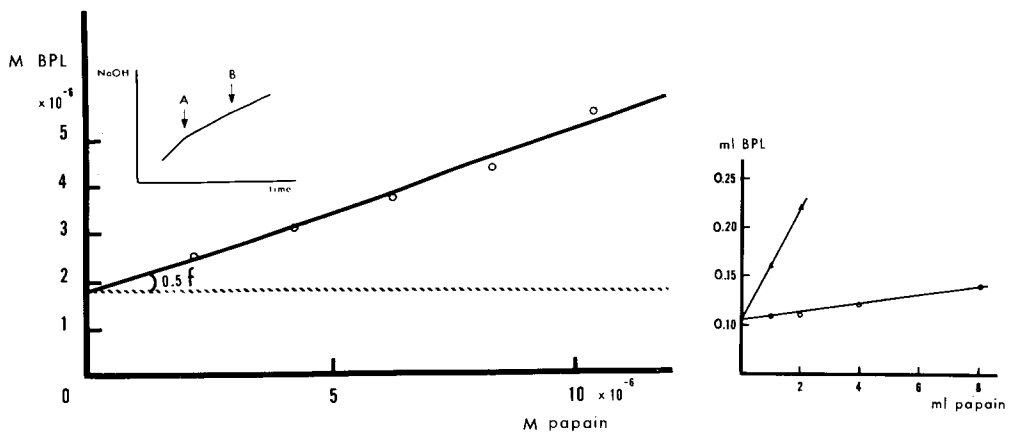


Fig. 1. Concentrations of BPL, necessary for 50% inhibition of BAEE hydrolysis by papain (Sample I), present in various quantities (see text). From the slope of the resulting straight line the active fraction of the papain sample, 0.62 in the present example, can be deduced. In the inset the method of determining $[I_t]$ (0.5) is illustrated, by showing a pH stat recorder trace. In this case the reaction mixture was composed of 0.4 ml papain solution $E_{280\ nm} = 2.86$, 0.01 M BAEE, 5 mM cysteine and 1 mM EDTA, final volume 10 ml, pH 4.3, 25°. At A 0.14 ml $1.66 \cdot 10^{-4}$ M BPL solution was added, at B 0.02 ml BPL. From A to B $i = 0.48$, after B $i = 0.52$, as can be deduced from the progress curve. Therefore $[I_t]$ (0.5) is about 0.15 ml at the papain concentration mentioned above.

Fig. 2. BPL binding to papain Sample III: ○—○, the amount in ml BPL, necessary for 50% inhibition of EDTA activated papain and △—△ in ml BPL, mecessary for 50% inhibition of EDTA–cysteine activated papain. For conditions see text.

had a turnover rate of 0.28 mole NaOH per mole papain per sec, *i.e.* 15% activity.

It is shown below that in Sample III only about 30% of the papain can bind BPL, which is equal to the percent activity. This proves that irreversibly blocked papain cannot bind BPL. As in our papain crystals obviously a minor fraction is activatable, the low occupancy of the inhibitors can already be explained.

Binding behavior of reversibly blocked papain

During the activity experiments it was noted that Sample III had a low activity in the presence of EDTA alone. This sample is very useful for demonstrating whether reversibly blocked papain (to be activated by cysteine, probably a mixed disulfide of cysteine and papain) can bind inhibitors. According to the turnover rate, EDTA activated papain of Sample III was 7% active. For four different concentrations of this papain solution (activated only by EDTA), the amount of BPL necessary for 50% inhibition was determined. The results are seen in Fig. 2. It is evident that also at rather high papain concentrations, there is still a limited binding of BPL to papain, indicating that only the remaining small active fraction did bind BPL. Due to the low binding capability it was only possible to make a rough calculation of the binding fraction of the sample. It showed that only 3–5% of the total papain quantity did bind BPL. The stock solution of Sample III was also investigated under full activation (with cysteine + EDTA). As is seen from Fig. 2 the BPL binding is now considerably better corresponding to an activity of about 30%, completely in accordance with the percentage, evaluated from the turnover rate of BAEE.

Binding behavior of mercuripapain

Papain activity can be inhibited by mercury salts, *e.g.* according to the reaction: Papain–SH + $HgCl_2$ → Papain–S–HgCl + HCl. The reaction is almost stoichiometric, due to the strong binding of $HgCl_2$ to the SH group. By addition of cysteine the mercury can be removed from the SH group. EDTA addition does not show this effect, as reported by SLUYTERMAN[16].

The binding properties of mercuripapain were also investigated. Moreover the mercuripapain offered an other possibility to determine the percentage of active papain in a sample.

Sample I (E at 280 nm = 3.28) was taken and its activity determined in the presence of EDTA. It was then active for 24%. Subsequently an amount of $5 \cdot 10^{-4}$ M $HgCl_2$ solution was added to the titration vessel, resulting in a decrease of hydrolytic activity. The addition of $HgCl_2$ was repeated twice. The obtained pH stat recorder trace is drawn in Fig. 3. In fact the papain–SH group is titrated in this way, with the BAEE hydrolysis as indicator. We calculated from the curve of Fig. 3 that $1.5 \cdot 10^{-8}$ mole $HgCl_2$ would be needed to inactivate the papain completely. As the total papain quantity, calculated from E at 280 nm is equal to $5.9 \cdot 10^{-8}$ mole, the activity percentage calculated from this titration corresponds to 26%. The activity percentages of the four investigated papain samples, are collected in Table II.

To determine, whether mercuripapain could bind BPL, we added to 6 ml of the papain solution, containing $8.9 \cdot 10^{-8}$ mole of active papain, 0.13 ml $5 \cdot 10^{-4}$ M ($= 6.5 \cdot 10^{-8}$ mole) $HgCl_2$ solution. The remaining $2.4 \cdot 10^{-8}$ mole active papain then should correspond to 7% of the total papain. The turnover rate of this papain solution (EDTA activated) indicated an activity of 9%.

TABLE II

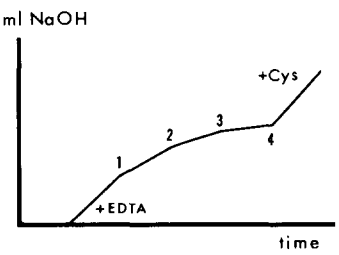
ACTIVITY OF FOUR PAPAIN SAMPLES, AS DETERMINED BY THREE DIFFERENT METHODS

C + E ,cysteine + EDTA activated; E, activated by EDTA alone.

Sample	*Turnover (%)*	*Hg^{2+} titration (%)*	*BPL binding (%)*
I, C + E	60		60
E	24	26	
II, C + E	33		
E	26	20	
III, C + E	30		30
E	7		3–5
IV, C + E	16		

The binding capacity of 2.4 and 6 ml of this papain solution with BPL was investigated. For 50% inhibition the requirements were, respectively, 0.12, 0.15 and 0.19 ml of the BPL solution. From these figures it can be calculated that approx. 9% of the total papain was capable of binding BPL, or in other words again only the remaining active fraction bound inhibitor.

We have used therefore three independent ways to determine the active fraction in a papain sample, activated by EDTA: (1) activity (turnover), (2) $HgCl_2$ titration, (3) BPL binding. In subsequent experiments additional proof of the non-binding of products to mercuripapain was obtained. To five 6-ml portions of papain solution (Sample I) were added various aliquots, 0.10, 0.13, 0.15, 0.20 and 0.22 ml $HgCl_2$, $5 \cdot 10^{-4}$ M. Then the binding properties of all these solutions were investigated. The amounts of BPL, necessary for 50% inhibition, can be read from Fig. 4. The percentages of activity, determined with the three methods mentioned above are summarized in Table III. During the calculations it became clear that the mercury titration was the least reliable method, due to the presence of EDTA which competes slightly



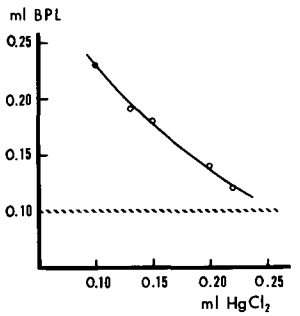


Fig. 3. Papain, titrated with $HgCl_2$. Shown is a pH stat trace of 0.01 M BAEE hydrolysis. First part: 1 mM EDTA added as activator. At 1, 2, 3, respectively, 0.01 ml, 0.005 ml and 0.005 ml $5 \cdot 10^{-4}$ M $HgCl_2$ was added. At 4, 6 mg of cysteine (5 mM) was added. The papain concentration was $5.9 \cdot 10^{-6}$ M (10 ml solution). Before 1 the EDTA activity and after 4 the cysteine EDTA activity can be calculated.

Fig. 4. ml of BPL solution, necessary for 50% inhibition of 6 ml portions of papain containing different amounts of $HgCl_2$ solution. *X*-axis represents ml of $5 \cdot 10^{-4}$ M $HgCl_2$ solution, added to 6 ml papain solutions (Sample I, *E* at 280 nm = 3.2).

TABLE III

ACTIVITY OF 6 ml OF PAPAIN (SAMPLE I, E AT 280 nm = 3.28), INACTIVATED BY VARIOUS AMOUNTS OF $5 \cdot 10^{-4}$ M $HgCl_2$

See text. The active fraction is calculated in three ways. First column: amount of added $HgCl_2$ solution. Second column: molar ratio of $HgCl_2$ to active papain assuming an activity of 24% without $HgCl_2$ addition. Column 3: expected activity if all added mercury would have occupied the SH group. Column 4: activity calculated from the turnover rate of BAEE hydrolysis. Column 5: activity deduced from BPL binding.

$HgCl_2$ added (ml)	*$HgCl_2$/ active papain*	*Expected activity (%)*	*Turnover (%)*	*BPL binding (%)*
0	0	24	24	
0.10	0.56	11	13	13
0.13	0.73	7	9	9
0.15	0.84	4	8	8
0.20	1.12	0	3	4
0.22	1.24	0	2	2

with papain for $HgCl_2$ (ref. 16). Only after the addition of an excess of $HgCl_2$, does the papain activity totally disappear. This is apparent from the values of Table III.

Check of Eqn. 3

Since $[I_f]$ (0.5) and $[I_b]$ (0.5) of BPL for papain Sample I are known, it is easy to calculate $[I_t]$ for any fractional inhibition for all papain concentrations of Sample I, using Eqn. 3. It will therefore be an interesting check of *Method 1* to see if in the experiment the predicted $[I_t]$ value actually will be observed. We thought it desirable to check our method. We prepared solutions of Sample I, E at 280 nm = 3.04 and calculated the quantities of BPL solution, necessary for inhibiting, respectively, 0.5, 1.0 and 1.5 ml papain solution to the extent of 20, 50 and 70%. The experimental check was made, using the conditions previously mentioned. The calculated amounts of BPL were added and the observed fractional inhibitions were compared with those calculated. As will be seen from Table IV, a very good agreement is found, indicating that our method is indeed reliable.

Method 2. Reaction of papain and iodoacetic acid + BAEE

First the alkylation reaction of iodoacetic acid and papain in the presence of

TABLE IV

CHECK OF EQN. 3

For details see text.

BPL (ml)	*0.5 ml papain*		*BPL (ml)*	*1.0 ml papain*		*BPL (ml)*	*1.5 ml papain*	
	i (calc.)	*i (obs.)*		*i (calc.)*	*i (obs.)*		*i (calc.)*	*i (obs.)*
0.05	0.20	0.20	0.07	0.20	0.18	0.09	0.20	0.21
0.15	0.50	0.49	0.20	0.50	0.50	0.25	0.50	0.50
0.28	0.68	0.69	0.37	0.70	0.70	0.44	0.70	0.71

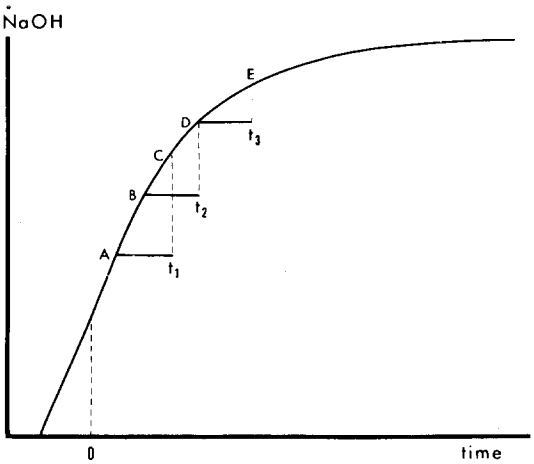


Fig. 5. Evaluation of the half life for reactions of alkylating agents with papain. Drawn is a pH stat curve, representing BAEE hydrolysis by papain. At $t = 0$, the alkylating agent was added. From the trace the half life of the reaction is determined as follows. Let the rate at C be half the rate at A, at D half the rate at B, at E half the rate at C. The half life must then be $t_1 = t_2 = t_3$ sec. This last equality is a good check for the quality of the curve.

varying concentrations of BAEE was examined. The conditions were: papain $1.36 \cdot 10^{-6}$ M, cysteine–EDTA, KCl as usual and, respectively, 0.01, 0.02 and 0.04 M BAEE. The total volume was 10 ml, the pH 4.3, the temperature 25° and the titration was with 0.0188 M NaOH. In all three cases at t = 0, 0.2 ml $1 \cdot 10^{-3}$ M iodoacetic acid was added. The half life of the inhibition reaction determined according to Fig. 5 in 0.01 M BAEE was 42 sec in 0.02 M BAEE 38 sec and in 0.04 M BAEE 39 sec corresponding to bimolecular rate constants of, respectively, 850, 920 and 900 $M^{-1} \cdot sec^{-1}$. From these values one can conclude that also iodoacetic acid reacts as fast with free papain as with the papain–BAEE complex.

Reaction of papain and iodoacetic acid + BAEE and BPL

The reaction conditions were altered. The papain concentration was as low as $0.68 \cdot 10^{-6}$ M. 10 ml of this papain solution cannot bind more than 0.01 ml $1.66 \cdot 10^{-4}$ M BPL solution. The BAEE concentration was 0.01 M; activators and KCl were present. Three runs were performed: (1) without BPL, (2) with 0.1 ml BPL solution, (3) with

TABLE V

HALF LIVES AND SECOND-ORDER RATE CONSTANTS OF THE IODOACETIC ACID REACTION WITH PAPAIN IN THE PRESENCE OF BPL

Correlation between $t_{\frac{1}{2}}$ and k: $k = 0.69/t_{\frac{1}{2}} \times 2.5 \times 10^{-6}$.

Conditions	$t_{\frac{1}{2}}$ *(sec)*	k_{obs} $(M^{-1} \cdot sec^{-1})$	k_{calc} $(M^{-1} \cdot sec^{-1})$
0.01 M BAEE, no BPL	270	1000	1000
0.01 M BAEE, $1.66 \cdot 10^{-6}$ M BPL	510	530	570
0.01 M BAEE, $3.32 \cdot 10^{-6}$ M BPL	780	360	360

0.2 ml BPL solution. In all three cases at $t = 0$, 0.025 ml $1 \cdot 10^{-3}$ M iodoacetic acid was added. The observed half lives are given in Table V. The rate constant in the absence of BPL is approx. 10% higher than previously determined, perhaps as a result of the slightly different experimental conditions. It is evident however that in the presence of BPL the rate constants of the alkylation reaction decrease, which means, that the papain–BPL complex is either not at all or less accessible for reaction with iodoacetic acid. If papain–BPL is totally inaccessible for iodoacetic acid, calculations can be made as to what the rate constants should be in the presence of different BPL concentrations. Since both papain–BAEE and free papain react at a rate of 1000 $M^{-1} \cdot sec^{-1}$ with iodoacetic acid, this rate must be reduced to $(v_i/v_0) \cdot 1000$ M^{-1}, where v_3 and v_0 are the hydrolysis rates in the presence and absence of BPL before addition of iodoacetic acid. The ratio's v_i/v_0 are 0.57 for 0.1 ml BPL and 0.36 for 0.2 ml BPL, deduced from the progress curves. One can expect k values of 570 and 360, respectively. Indeed approximately these values were experimentally observed (see Table V). So BPL–papain is totally inaccessible to reaction with iodoacetic acid. This is also demonstrated in Fig. 6, where k is plotted against v_i/v_0. The two experimentally

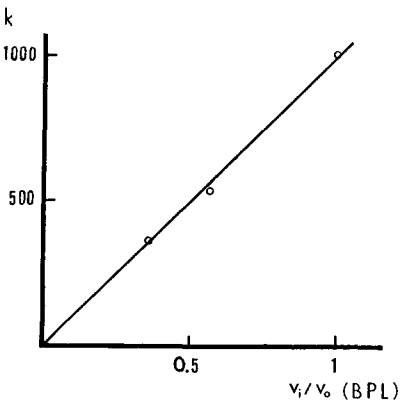


Fig. 6. Change of the second-order rate constant of iodoacetic acid reaction with papain (k), against v_i/v_0 (BPL). v_0 rate of BAEE hydrolysis, v_i rate of BAEE hydrolysis (same concentration BAEE) in the presence of added BPL.

proven facts that (1) SH-blocked papain cannot bind BPL and that (2) papain–BPL complex cannot react with iodoacetic acid, are probably equivalent.

The fact, that the extremely good inhibitor BPL binds only to active papain, does not imply that all competitive inhibitors will have that property. It is therefore advisable to investigate the binding properties of poorer binding inhibitors. The product inhibitor benzoylarginine with a K_i (at pH 4.3) of 0.03 M belongs in this group.

Reaction of papain and iodoacetic acid + BAEE and benzoylarginine

We investigated the effect of the poorer inhibitor benzoylarginine on the alkylation reaction. The experimental conditions and results are given in Table VI. It is evident that the alkylation of papain decreases in the presence of benzoylarginine.

TABLE VI

HALF LIVES AND SECOND-ORDER RATE CONSTANTS OF IODOACETIC ACID REACTION WITH PAPAIN, IN THE PRESENCE OF BENZOYLARGININE

Conditions: (a) 0.03 M BAEE, cysteine EDTA–KCl as usual $1.1 \cdot 10^{-6}$ M papain, pH 4.3, 25°, total volume 10 ml. At $t = 0$, 0.05 ml 1 mM iodoacetic acid added. (b) Same as (a), but in addition 0.03 M benzoylarginine. (c) Same as (a) but in addition 0.05 M benzoylarginine and $1.66 \cdot 10^{-6}$ M papain.

Run	$t_{\frac{1}{2}}$ *(sec)*	k_{obs} $(M^{-1} \cdot sec^{-1})$	k_{calc} $(M^{-1} \cdot sec^{-1})$
0.03 M BAEE	130	1100	1100
0.03 M BAEE, 0.03 M benzoylarginine	180	770	730
0.03 M BAEE, 0.05 M benzoylarginine	300	460	490

It is again possible to derive the reduction in alkylation rate, assuming that benzoylarginine–papain cannot react with iodoacetic acid. The calculated rate constants are in close agreement with the observed values (Table VI).

Reaction of papain and iodoacetamide + BAEE and benzoylarginine.

An objection can still be raised against the experiments with iodoacetic acid. The negative charge of the competitive inhibitors, bound to the protein, would prevent the approach of the also negatively charged iodoacetic acid. Perhaps a neutral reagent could indeed react with the active site, occupied by products. Therefore we repeated our experiments with iodoacetamide. The experimental conditions and results are given in Table VII.

TABLE VII

SECOND ORDER RATE CONSTANT OF IODOACETAMIDE REACTION WITH PAPAIN, IN THE PRESENCE OF BENZOYLARGININE

Conditions: (a) 0.03 M BAEE, $1.1 \cdot 10^{-6}$ M papain. At $t = 0$ addition of 0.05 ml 0.1 M iodoacetamide added. (b) 0.06 M BAEE, $0.55 \cdot 10^{-6}$ M papain. At $t = 0$ 0.025 ml 0.1 M iodoacetamide added. (c) 0.03 M BAEE, 0.03 M benzoylarginine, $1.65 \cdot 10^{-6}$ M papain. At $t = 0$ 0.025 ml iodoacetamide added. (d) 0.03 M BAEE, 0.05 M benzoylarginine, $1.65 \cdot 10^{-6}$ M papain. At $t = 0$ 0.05 ml iodoacetamide added. Activators, KCl present as usual, 25°, pH 4.3.

Run	k_{obs} $(M^{-1} \cdot sec^{-1})$	k_{calc} $(M^{-1} \cdot sec^{-1})$
a	10	10
b	10	
c	7	7.1
d	6	5.8

Again, in the presence of BAEE alone, the alkylation rate of iodoacetamide does not change, but in the presence of benzoylarginine decreases. In the latter case benzoylarginine–papain is again totally inaccessible to iodoacetamide.

In passing it may be noted that the reaction rate for iodoacetic acid is a hundred times higher than for the amide. A similar phenomenon was observed by WALLENFELS AND EISELE[17], who measured the alkylation rate of L- and D-α-iodopropionic acid and amide. The alkylation reactions themselves appear to be very interesting in view of the current thoughts on the reaction mechanism of the papain-catalyzed hydrolysis.

It could be possible that the used iodoacetamide solution was contaminated by small amounts of iodoacetic acid. Iodoacetamide was therefore checked carefully for contamination with iodoacetic acid by titration with 0.0188 M NaOH. The iodoacetic

acid content was found to be far less than 1%. Therefore we are confident, that the alkylation rates, measured with iodoacetamide are indeed due to this reagent.

DISCUSSION

Our results have important consequences for the X-ray studies of crystalline papain–product complexes. In those studies one must work with crystals which are both activated and contain a very high SH content (of Cys 25), otherwise the occupancy with products will be low. Furthermore it is curious that in benzoylarginine–papain the alkylation reactions are prevented, while this is not the case for BAEE–papain. One would expect these closely related substances to have similar binding properties and one might even predict the reverse, considering the bulkiness of the BAEE molecule compared to benzoylarginine. An important question is therefore whether the observed binding constant K_i for a product is due to a real product–enzyme complex or to an acylenzyme, originating from the reaction:

$$\text{Papain–SH} + \text{R–COOH} \rightarrow \text{Papain–S–COR} + H_2O$$

From our experiments alone we cannot conclude that binding of product is in fact acylation of the enzyme, but it is certainly worthwhile to investigate this possibility further. Some support is found in the literature[18,19].

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