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THE RATE-LIMITING REACTION IN PAPAIN ACTION AS DERIVED FROM THE REACTION OF THE ENZYME WITH CHLOROACETIC ACID

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SUMMARY

Chloroacetic acid reacts with the essential thiol group of papain (EC 3.4.4.10) and inactivates the enzyme. The rate of inactivation, k, at pH 6.0, 25°, was determined in the absence and presence of the substrates benzoylarginine ethyl ester (BAEE) and benzoylglycine ethyl ester (BGEE). The rate was found to be independent of BAEE concentration and to decrease with increasing BGEE concentration. Extrapolation to infinite BGEE concentration yielded $k = 0$. These results indicate that (a) acylation of the thiol group is the rate-limiting step in BAEE hydrolysis, (b) deacylation is the rate-limiting step in BGEE hydrolysis.

The thiol group of papain is 30 000 times more reactive towards chloroacetic acid than is the thiol group of cysteine at pH 6.

v_{max} values were determined for four benzoylarginine derivatives, the methyl, ethyl and propyl esters and the amide. These values were found to differ by no more than a factor of three. It is pointed out that caution should be exerted in interpreting v_{max} data in terms of rate-limiting steps.

INTRODUCTION

It has been known for some time that chymotrypsin and other esterases exert their action in three steps, with two intermediates: the enzyme substrate complex and the acylenzyme. In the latter intermediate the OH group of the serine in the active site is attached to the acid part of the substrate in an ester linkage. In the case of papain (EC 3.4.4.10), which carries an essential SH group, an intermediate acylenzyme had been postulated[1], in which the acyl group is linked to the SH group in a thiol ester linkage. This was definitely established by LOWE AND WILLIAMS[2], who used an elegant spectroscopic method involving methyl thionohippurate as substrate. Similar results were obtained by BENDER AND BRUBACHER[3], using the nonspecific substrate cinnamoyl imidazole.

Abbreviations: BA, benzoylarginine; BAA, benzoylarginine amide; BAME, benzoylarginine methyl ester; BAEE, benzoylarginine ethyl ester; BAPE, benzoylarginine *n*-propyl ester; BG, benzoylglycine; BGEE, benzoylglycine ethyl ester; CAA, chloroacetic acid.

Values of K_m and k_{cat} were determined for a number of esters of benzoylglycine[4] and of carbobenzoxyglycine[5]. For most esters in each group the values of k_{cat} were found to be virtually equal. This was interpreted as indicating that the deacylation of the *S*-acylpapain intermediate is the rate-limiting step in the hydrolysis. The same interpretation for benzoylarginine (BA) derivatives was suggested by SMITH[1], who observed equal k_{cat} values for benzoylarginine ethyl ester (BAEE) and benzoylarginine amide (BAA) at 37°.

Although the data for benzoylglycine and carbobenzoxyglycine derivatives seem conclusive, there are some indications that the hypothesis concerning BAEE and BAA is not the right one. The temperature dependence and hence the activation enthalpy of k_{cat} at pH 6 are greater for BAA[6] than for BAEE[7]. For a common rate-limiting intermediate these values, of course, should be equal. Furthermore WHITAKER AND BENDER[8] report a k_{cat} value for BAA which is half the value for BAEE at 25°. Finally, it has been reported in a previous paper[9] that BAEE combines with the active site of nonactivated papain. The resulting complex has a dissociation constant equal to the K_m value of activated enzyme. This has been interpreted as indicating that the acylation and not the deacylation is the rate-limiting step in the hydrolysis of BAEE.

In order to find out whether acylation or deacylation is rate-limiting, the usual procedure of comparing the k_{cat} values of a few esters of BA was first attempted, but this did not lead to decisive results. It proved to be possible to arrive at a definite conclusion in a novel manner, by studying the rate of inactivation of papain by chloroacetic acid in the presence of various concentrations of substrate.

EXPERIMENTAL

Materials

For all kinetic experiments mercuripapain was employed, prepared by a method described recently[10]. Benzoylglycine ethyl ester (BGEE) was prepared by esterification of benzoylglycine (BG)[11]. Benzoylarginine *n*-propyl ester (BAPE) was prepared by transesterification of BAEE, 5 g of which was dissolved in 160 ml dry propanol. At 0° HCl gas was passed through until saturation. The mixture was stored overnight at 4°. The propanol was removed *in vacuo*. The residue was dried over potassium hydroxide pellets. The whole procedure was repeated once more. The dry residue was twice crystallized from chloroform–ether. Yield 5.0 g, m.p. 146°. Analysis: C, 53.5%; H, 7.2%; N, 15.2%; Cl, 10.1%. Calculated: C, 54.0%; H, 7.1%; N, 15.7%; Cl, 10.0%.

All other compounds were commercial preparations: BA and benzoylarginine methyl ester (BAME) (Mann Research Laboratories, New York), BAA and BAEE (Calbiochem, Lucerne, Switzerland). The purity of chloroacetic acid (CAA), m.p. 61–63° (Brocades and Stheeman, Amsterdam) was checked by titration.

Kinetic measurements

For the reaction of papain with CAA in the presence of substrate, the pH-stat equipment, the conditions and procedures were those employed in the investigation of the reaction of papain with cyanate[10]. The rate of inactivation in the absence of substrate was determined by means of a discontinuous method. To a mixture of approx. 10^{-6} M papain, 5 mM cysteine and 1 mM EDTA, 0.3 M KCl, pH 6.0, 25°, in

the reaction vessel of the pH stat, a 0.1-ml aliquot of 5.2 mM CAA (adjusted to pH 6.0) was added. Total volume was 9.6 ml. After the desired interval (0, 2, 4, 6, 8, 10 or 12 min) 0.5 ml of 0.2 M BAEE (also adjusted to pH 6.0) was added. The initial slope of the progress curve on the recorder of the pH stat indicated the residual activity after the interval concerned. A semilogarithmic plot of the residual activity *versus* time gave a straight line, the slope of which provided the reaction rate constant.

Michaelis–Menten parameters were measured under the usual conditions of 5 mM cysteine, 1 mM EDTA, 0.3 M KCl, pH 6.0, 25° or 40°, Substrate concentrations were 15–120 mM BAME, BAEE, or BAPE and 5–40 mM BGEE. The results were plotted in Lineweaver–Burk plots, 12 to 14 points for each plot. Regression lines and standard deviations were calculated from least squares on a computer.

For v_{max} determinations the papain solutions were carefully standardized on 120 mM BAEE at 25°, pH 6.0.

Rate measurements on 0.1 M BAA were carried out on a Perkin–Elmer polarimeter 141, provided with a recorder, adjusted to 0.030° at full-scale deflection, at $\lambda = 578$ mμ. For activation DL-cysteine was used. Buffer was 0.02 M phosphate (pH 6.0). For extrapolation to infinite substrate concentration, K_m was taken to be 33.3 mM (ref. 8). Rotation at 578 mμ + 0.203° ± 0.005 for 0.105 M BAA and +0.278 ± 0.006 for 0.0536 M BA. From this a change of rotation of 3.26° per mole BAA hydrolyzed can be calculated (per dm).

Thiol titrations

In order to check the specificity of the action of CAA on active papain, 5 ml of approx. 1% gel-filtered papain solution in 0.02 M acetate (pH 5.0)–1 mM EDTA, was made 5 mM cysteine and 0.07 to 4 mM in CAA and stored at room temperature. After 15–30 min, when the desired decrease in activity was nearly obtained, the reaction mixture was gel-filtered once more, in order to remove activator and CAA. The resulting solution of partially inactivated papain was used for activity assay (0.1-ml aliquots) and for thiol titration (2-ml aliquots), both in duplicate. For the thiol titrations a small amperometric apparatus was used[10]. The titration medium contained 50 mM imidazole (pH 7.0)[12], 10 mM KCl, 1 mM EDTA and 5 mM Na_2SO_3. Protein concentration was 0.2–0.5%. Total volume about 3 ml, titrated with 2.0 mM $AgNO_3$.

RESULTS

Maximum velocity of a few BA derivatives

The kinetic parameters of BAA and three esters of BA are given in Table I. If the deacylation were the rate-limiting step, all k_{cat} values should be equal. In fact they are not. Although the differences between the esters are small, they are experimentally significant, especially the difference between the k_{cat} values of BAME and BAPE. The interpretation, however, is uncertain, since the results might be interpreted to indicate that the rates of acylation and deacylation are about equal in the case of BAA and that the rate of deacylation is almost rate-limiting in the case of the esters.

More decisive results were obtained by the experiments to be described in the next section.

TABLE I

KINETIC CONSTANTS OF PAPAIN-CATALYZED HYDROLYSIS OF BA DERIVATIVES AT pH 6.0, 25°

Substrate	k_{cat}	K_m *(mM)*
BAME*	1.15 ± 0.03	6.8 ± 0.4
BAEE*	1.00** ± 0.02	18.0 ± 1.0
BAPE	0.83 ± 0.025	19.0 ± 1.6
BAA	0.5 − 0.6***	33.3 ± 3.4***
BAA	0.41 ± 0.04	—

* *cf.* ref. 13.
** By definition.
*** At pH 5.2, Table I of ref. 8 (*cf.* ref. 6).

Reaction with chloroacetic acid

Halogenacetic acids and amides are known to be particularly reactive with thiol groups of proteins. Papain has been subjected to reaction with bromoacetic acid and iodoacetic acid[14] and the related enzyme ficin to reaction with chloroacetamide and iodoacetamide[15]. In all cases specific reaction with the essential thiol group has been established. One may therefore expect the same specificity for chloroacetic acid and papain. A virtually proportional decrease in activity (towards BAEE) and in thiol content (estimated by amperometric argentometric titration) has indeed been found (Fig. 2, inset). It may therefore be concluded that the main reaction occurs according to:

$$E\text{SH} + \text{Cl–CH}_2\text{–COOH} \rightarrow E\text{S–CH}_2\text{–COOH} + \text{HCl} \qquad (1)$$

and that any other reaction, if present, is either much slower or occurs at non-essential parts of the enzyme.

Since the concentration of the reagent considerably exceeded the enzyme concentration in all experiments, a first-order reaction is to be expected:

$$\ln [E_a]/[E_0] = -k[C]\,t \qquad (2)$$

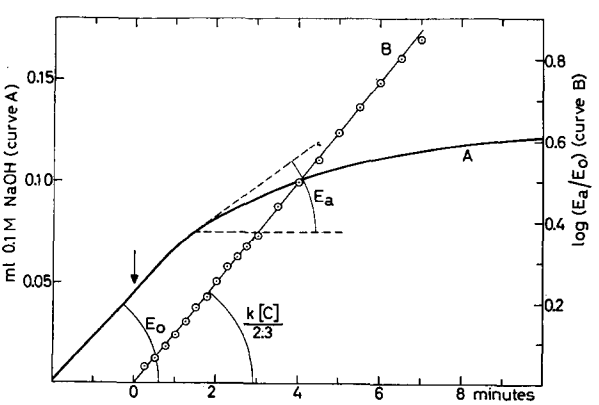

Fig. 1. Curve A. Copy of a progress curve of the hydrolysis of BGEE (10 mM) by papain previous to and after addition of 0.1 ml 0.316 M CAA (moment indicated by arrow). Conditions: approx. 10^{-6} M papain, 0.3 M KCl, pH 6.0, 25°, total volume 10 ml. Curve B. Semilogarithmic plot of the data of Curve A.

in which $[E_0]$, $[E_a]$ and $[C]$ denote the concentrations of initial enzyme, active enzyme and CAA, respectively. The following convenient procedure for studying the rate of inactivation of papain in the presence of various concentrations of substrate was adopted (*cf.* ref. 10). The desired concentrations of substrate (BAEE or BGEE), papain and activators were put into the reaction vessel of a pH-stat and the progress of the hydrolysis at pH 6.0 traced on the recorder. After the reaction had proceeded for 2 min, 0.1 or 0.2 ml of CAA solutions, previously adjusted to pH 6.0, was added (final concentration 1.6–6 mM). Inactivation was evident from the gradual decrease in slope of the progress curve (Fig. 1, Curve A). Tangents on the progress curve were drawn at 0.25-min or 0.5-min intervals. The slopes of these tangents, proportional to the concentration of active enzyme E_a at the moments concerned, were (if necessary) corrected for the decrease in substrate concentration during the run in the manner described in an earlier paper[9]. Plots of log $[E_a]/[E_0]$ *versus* time were found to be linear, as shown by the example in Fig. 1, Curve B. The adherence to Eqn. 2 was further checked by examining the effect of CAA concentration. Values of k were found to be independent of reagent concentration in the range of 6–40 mM in the presence of 0.04 M BGEE and in the range of 2–12 mM in the presence of 0.015 M BGEE.

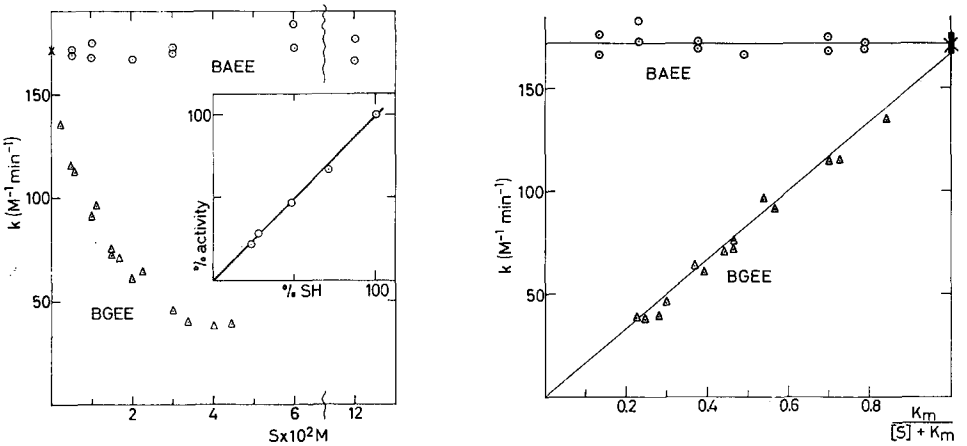

Fig. 2. The effect of substrate concentration on the inactivation rate constant. ○, BAEE; △, BGEE; ×, in the absence of substrate. Inset: proportionality of activity and thiol content; as a percentage of initial values.

Fig. 3. Plot of inactivation rate k *versus* $K_m/([S] + K_m)$. ○, BAEE; △, BGEE; ×, in the absence of substrate; the bar on the right-hand ordinate indicates the mean value and standard error of all values in the presence of BAEE.

Inactivation in the absence of substrate was determined by a discontinuous method described in the experimental part.

Values of k were determined at a number of substrate concentrations: 5–120 mM BAEE and 2.5–44 mM BGEE. The results show a striking difference between the two substrates (Fig. 2). BAEE does not affect the inactivation rate*, BGEE strongly inhibits the reaction.

* A few measurements were carried out at pH 5 and pH 7. Rate constants were equal to those at pH 6 and independent of BAEE concentration.

The following considerations show how to extrapolate to infinite substrate concentration. As mentioned in INTRODUCTION, the papain-catalyzed hydrolysis proceeds according to the three-step mechanism

$$E + S \overset{1}{\rightleftharpoons} ES \overset{2}{\rightarrow} ES' + P_1 \overset{3}{\rightarrow} E + P_2 \tag{3}$$

in which ES denotes the enzyme substrate complex and ES' the acyl enzyme. Total active enzyme E_a is thus present in three states: E, ES and ES'. In ES' the thiol group is covalently linked to the acyl part of the substrate and therefore cannot take part in the reaction with CAA. Hence the overall reaction consists of two terms:

$$k[E_a] = k_f[E] + k_c[ES] \tag{4}$$

in which k_f and k_c denote the inactivation rate constants of E and of ES.

It can be calculated from a steady-state treatment of Eqn. 3 that:

$$[ES'] = (k_2/k_3)\,[ES] \text{ and } [E] = [E_a]K_m/([S] + K_m)$$

These equations, combined with the conservation equation $[E_a] = [E]+[ES]+[ES']$, introduced into Eqn. 4 yield:

$$k = \left(k_f - \frac{k_c\,k_3}{k_2 + k_3}\right)\frac{K_m}{[S] + K_m} + \frac{k_c\,k_3}{k_2 + k_3} \tag{5}$$

Eqn. 5 shows that a plot of k *versus* $K_m/([S]+K_m)$ should yield a straight line, as was in fact observed (Fig. 3). Extrapolation to $[S]\rightarrow 0$ (*i.e.* $K_m/([S]+K_m) = 1$) yields $k = k_f$, extrapolation to $[S]\rightarrow\infty$ (*i.e.* $K_m/([S]+K_m) = 0$) yields $k = k_c k_3/(k_2+k_3)$.

In the case of BAEE ($K_m = 18$ mM) the line is a horizontal one (Fig. 3). The mean value of all k values determined in the presence of BAEE, $k = 172\pm5$, is in excellent agreement with the value determined in the absence of substrate, $k_f = 171$.

Since the line is horizontal, extrapolations to zero and to infinite substrate concentrations yield equal k values, *i.e.* $k_f = k_c k_3/(k_2+k_3)$. A theoretically possible explanation is that k_c is higher than k_f by a factor which is exactly equal to $(k_2+k_3)/k_3$. Such a compensation is very unlikely. Therefore one may conclude that $k_c = k_f$ and that $k_2 \ll k_3$, *i.e.* the acylation step is rate-limiting and not the deacylation step.

In the case of BGEE ($K_m = 13$ mM) the straight line extrapolates at $[S] = 0$ to virtually the same value of k_f reported above. At $[S]\rightarrow\infty$, however, extrapolation yields $k_c k_3/(k_2+k_3) = 0$. Theoretically this might indicate that k_c is zero. However, this explanation is very unlikely because in the case of the larger substrate BAEE k_c is certainly not zero, as shown above. Hence one may conclude that $k_2 \gg k_3$, *i.e.* in the case of BGEE the deacylation step is rate-limiting and not the acylation step. This is the same conclusion arrived at by LOWE AND WILLIAMS[4] (*cf.* INTRODUCTION). Thus two completely different experimental approaches lead to the same conclusion as far as BGEE is concerned.

Qualitatively the effects of BAEE and BGEE can be most readily understood as follows. Active enzyme molecules can be present as E, ES and ES'. At $[S]\rightarrow\infty$ $[E] = 0$ with both substrates. At $[\text{BAEE}]\rightarrow\infty$ virtually all enzyme molecules are present solely as ES (which happens to be as reactive towards CAA as is E), because ES' immediately decomposes into E *plus* P_2 and therefore is not present in a perceptible concentration. Hence $k_3 \gg k_2$, for BAEE. At $[\text{BGEE}]\rightarrow\infty$ all enzyme molecules are

present solely as ES' (which is unreactive towards the reagent) because ES is too quickly converted into ES' to be present in a perceptible concentration. Hence $k_2 \gg k_3$ for BGEE.

In order to compare the reactivities of the thiol groups of papain and of cysteine the reaction rate of the latter was measured in the pH-stat, by titration of the HCl evolved during the reaction (*cf.* Eqn. 1). The conditions were again pH 6.0, 25° and 0.3 M KCl. Both cysteine and CAA were 50 mM, titrated with 0.1 M NaOH, The rate constant derived from the initial reaction velocity was found to be $k = 5.3 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$ $M^{-1} \cdot min^{-1}$. Hence the reactivity of the thiol group of papain towards CAA is 32 000 times greater than is the reactivity of the thiol group of free cysteine. This ratio apparently is even more marked than in the reaction of KCNO (ref. 10).

DISCUSSION

There are now two sets of data available from which one may conclude that with BAEE the acylation is the rate-limiting step: (1) the equality of the rates of inactivation of papain by CAA in the presence of BAEE and in the absence of it, as discussed in this paper; (2) the equality of K_m of the active enzyme and K_s of the binding of BAEE to inactive papain, both at pH 6 and at pH 4, as discussed in a previous paper[9] (*cf.* INTRODUCTION to this paper).

In each case separately one might argue that these equalities are due to coincidental cancellation of opposing effects. However, it is extremely unlikely that such cancellations occur in both cases, especially as two different types of reaction were studied: an irreversible reaction in the first case and an adsorption equilibrium in the second case.

Two more sets of data fit nicely into the present picture and thus tend to confirm the conclusion:

(1) The effect of temperature (Table II). The K_m value of BAEE increases only slightly with temperature, as is to be expected of an ES complex which has an only slightly lower energy content than its component parts. The K_m value of BGEE, on the other hand, increases markedly with temperature. A similar observation has been made by LOWE AND WILLIAMS[4] on the methylester of benzoylglycine. In the case of BAEE $K_m = k_{-1}/k_1 = K_s$, but in the case of BGEE, where k_3 is rate-limiting, $K_m = K_s k_3/k_2$. Thus in the latter case the temperature dependence of k_3 and k_2 is also involved. If it is assumed that the temperature dependence of K_s, k_2 and k_3 is approxi-

TABLE II

EFFECT OF TEMPERATURE ON MICHAELIS–MENTEN PARAMETERS AT pH 6.0

Substrate	*Temp.*	K_m *(mM)*	k_{cat}	*Reaction step*
BAEE	25°	18	1.0*	k_2
BAEE	40°	20	1.75	k_2
BGEE	25°	13	0.165	k_3
BGEE	40°	21	0.39	k_3

* By definition.

mately the same for both substrates, one can calculate K_m of BGEE at 40° with the equation:

$$(K_m)^{G}_{40} = (K_m)^{G}_{25} \frac{(K_m)^{A}_{40}}{(K_m)^{A}_{25}} \frac{(k_3)^{G}_{40}}{(k_3)^{G}_{25}} \frac{(k_2)^{A}_{25}}{(k_2)^{A}_{40}}$$

The superscripts A and G denote the parameters of BAEE and BGEE, respectively, and the subscripts the temperature. Using the data of Table II, one calculates $(K_m)^{G}_{40}$ = 19.5 mM; the value actually found was 21 mM. This is a fair agreement.

(2) It has been observed that in 15% (by vol.) methanol the maximum velocity of the hydrolysis of BAEE is 33% lower than in water[16]. Taking into account that methanol is 5.6 times more reactive than water in the papain-catalyzed conversion of BAA[16], one can calculate* a decrease in v_{max} of the hydrolytic reaction of BAEE of 31% if $k_2 \ll k_3$ or 15% if $k_2 \gg k_3$. The former value is very near the observed one.

Another example of a different rate-limiting step for the esters of two substrates with a different acyl residue, is thrombine. For benzoylarginine esters deacylation is probably rate-limiting, for toluenesulphonylarginine esters the acylation[17].

WHITAKER AND BENDER[8] determined K_m and k_{cat} of papain-catalyzed hydrolysis of BAEE and BAA as a function of pH by difference spectroscopy. They concluded from their results that k_3 is rate-limiting in the case of BAEE and k_2 is partially so in the case of BAA. Their main argument for a different rate-limiting step in the two cases is that the alkaline branches of the k_{cat} *versus* pH curves for the two substrates seem to be governed by different pK values. However, the limited accuracy of their data, especially those of BAA above pH 7 (Fig. 3 of ref. 8), does not warrant such a conclusion.

Furthermore they assume that from pH 5 upwards K_s and k_3 are pH-independent. They ascribe changes in K_m and k_{cat} entirely to pH dependence of k_2. In doing so they derive certain values for K_s, k_2, k_3 and a pK value for k_2. However, within experimental error a pK value of 8.7 for K_m (lim. 13 mM) and of 9.3 for k_{cat} (lim. 15.5

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* In a mixture of water and methanol the last step of Eqn. 3 is replaced by the two competing reactions:

$$ES' \xrightarrow{k_3'\,[H_2O]} E + \text{acid}$$
$$ES' \xrightarrow{k_4'\,[\text{methanol}]} E + \text{ester}$$

Steady state calculation shows that the maximum rate of acid liberation as measured by the pH-stat (*cf.* ref. 25) is:

$$k_{cat} = \frac{k_2 k_3'[H_2O]}{k_2 + k_3'[H_2O] + k_4'[\text{methanol}]}$$

The ratio R of k_{cat} in water–methanol mixtures and in pure water (concentration $[H_2O]_0$) is:

$$R = \frac{[H_2O]}{[H_2O]_0} \frac{k_2 + k_3'[H_2O]_0}{k_2 + k_3'[H_2O] + k_4'[\text{methanol}]}$$

This reduces to $R = [H_2O]/[H_2O]_0 = 0.85$ in 15 vol.% methanol if Steps 3 *plus* 4 are rate-limiting (*i.e.* $k_2 \gg k_3'\,[H_2O] + k_4'[\text{methanol}]$) and to $R = [H_2O]/([H_2O] + (k_4'/k_3')\,[\text{methanol}])$ if Step 2 is rate-limiting. In 15 vol.% methanol [methanol] = 3.7 M and $[H_2O]$ = 47 M. In the hydrolysis of BAA the ratio of k_4'/k_3' is 5.6. This yields $R = 0.69$ in 15% methanol if Step 2 is rate-limiting.

sec^{-1}) fit their data just as well*, without any further assumptions regarding these experimental parameters. Hence these experimental data alone do not allow any conclusion about the identity of K_m and k_{cat} and hence no conclusion about which step is rate-limiting. Therefore the experimental data of WHITAKER AND BENDER do not actually conflict with our results.

It may at first seem surprising that there is so little difference in the k_2 values of the four BA derivatives of Table I, since in OH^--catalyzed hydrolysis of esters and amides there are large differences in rate of hydrolysis, as shown by the examples of Table III, second column. However, two points are to be considered. First of all, in

TABLE III

RATE CONSTANTS OF ACID- AND BASE-CATALYZED HYDROLYSIS OF ESTERS AND AMIDE OF ACETIC ACID AT 25°

	OH^--catalyzed			*H^+-catalyzed*		
	k $(M^{-1} \cdot sec^{-1})$	*Ratio*	*Ref. No.*	*k* $(M^{-1} \cdot sec^{-1})$	*Ratio*	*Ref. No.*
Methyl	$15.2 \cdot 10^{-2}$	1.6	18	$1.35 \cdot 10^{-4}$*	1.2	20
Ethyl	$9.8 \cdot 10^{-2}$*	1.0	19	$1.12 \cdot 10^{-4}$	1.0	22
Amide	$4.7 \cdot 10^{-5}$*	$5.0 \cdot 10^{-4}$	21	$6.6 \cdot 10^{-6}$	0.06	20
Benzyl**	$7.0 \cdot 10^{-2}$	1.0	23	$3.1 \cdot 10^{-5}$	1.0	23
Phenyl**	0.54	8	23	$2.8 \cdot 10^{-5}$	0.9	23
m-Nitrophenyl**	5.5	80	23	$1.9 \cdot 10^{-5}$	0.6	23

* Calculated for 25° from the available data.
** In 60% (by vol.) acetone.

papain the attacking agent is not the nucleophilic OH^-, but a thiol group is involved (possibly in non-ionized state). Secondly the activation energies in enzyme reactions are generally lower than those of nonenzymic reactions. Since differences in rates between related compounds like esters are the result of a delicate balance between the effects of activation energy and activation entropy, one cannot make any certain predictions, if at least one of these factors is changed, on comparing different types of catalysis. This is illustrated by the data in Table III. In acid-catalyzed hydrolysis the differences are considerably smaller than in base-catalyzed hydrolysis. Other such examples can be given[24]. The data in the lower half of Table III even show a reversal of the differences. Therefore one need not be surprised that papain, in the case of specific substrates, may not always "mind" very much which derivative it hydrolyses. The data suggest that papain action has something in common with H^+-catalyzed hydrolysis. The inference is that, in comparing k_{cat} values of various esters and amides of one acid, even small differences may be significant. Strictly speaking, even complete equality of k_{cat} values does not absolutely *prove* that deacylation is rate-limiting, although such a conclusion is the most likely one and therefore is justified in most cases.

In a previous paper a similar kind of investigation, of the reaction of papain

* Including an almost straight line in their $1/K_m$ *versus* k_{cat}/K_m plot (Fig. 5 of ref. 8). Hence the interpretation of such a plot proposed by WHITAKER AND BENDER should be regarded with caution.

with cyanate was reported[10]. This (reversible) reaction too occurs with the thiol group. In both cases the thiol group of papain is far more reactive than is the thiol group of cysteine: 3000 times in the case of cyanate and even 30 000 times in the case of CAA. Most remarkable is the effect of BAEE in these two cases: a partial protection of papain against cyanate and no protection against CAA. This difference is not simply a matter of charge, since both reagents are ionized at pH 6, nor of size, since the larger molecule (CAA) is not hampered by substrate whereas the smaller one is. A different type of reaction mechanism is possibly involved. These phenomena deserve further investigation.

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REFERENCES

1 E. L. SMITH, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 4, Academic Press, New York, 2nd ed., 1960, p. 133.
2 G. LOWE AND A. WILLIAMS, *Biochem. J.*, 96 (1965) 189.
3 M. L. BENDER AND L. J. BRUBACHER, *J. Am. Chem. Soc.*, 86 (1964) 5333.
4 G. LOWE AND A. WILLIAMS, *Biochem. J.*, 96 (1965) 199.
5 J. F. KIRSCH AND M. IGELSTRÖM, *Biochemistry*, 5 (1966) 783.
6 A. STOCKELL AND E. L. SMITH, *J. Biol. Chem.*, 227 (1957) 1.
7 E. L. SMITH AND M. J. PARKER, *J. Biol. Chem.*, 233 (1958) 1387.
8 J. R. WHITAKER AND M. L. BENDER, *J. Am. Chem. Soc.*, 87 (1965) 2728.
9 L. A. Æ. SLUYTERMAN, *Biochim. Biophys. Acta*, 113 (1966) 577.
10 L. A. Æ. SLUYTERMAN, *Biochim. Biophys. Acta*, 139 (1967) 439.
11 L. A. Æ. SLUYTERMAN, *Biochim. Biophys. Acta*, 85 (1964) 305.
12 L. A. Æ. SLUYTERMAN, *Anal. Biochem.*, 14 (1966) 317.
13 A. N. GLAZER, *J. Biol. Chem.*, 241 (1966) 3811.
14 A. LIGHT, *Biochem. Biophys. Res. Commun.*, 17 (1964) 781.
15 M. R. HOLLAWAY, A. P. MATHIAS AND B. R. RABIN, *Biochim. Biophys. Acta*, 92 (1964) 111.
16 L. A. Æ. SLUYTERMAN, *Biochim. Biophys. Acta*, 139 (1967) 418.
17 E. F. CURRAGH AND D. T. ELMORE, *Biochem. J.*, 93 (1964) 163.
18 R. A. FAIRCLOUGH AND C. N. HINSHELWOOD, *J. Chem. Soc.*, (1937) 538.
19 E. A. MOELWYN-HUGHES, *The Kinetics of Reactions in Solution*, The Clarendon Press, Oxford, 1947, p. 124.
20 E. A. MOELWIJN-HUGHES, *The Kinetics of Reactions in Solution*, The Clarendon Press, Oxford, 1947, p. 321.
21 J. PACKER, A. L. THOMSON AND J. VAUGHAN, *J. Chem. Soc.*, (1955) 2601.
22 R. P. BELL, A. L. DOWDING AND J. A. NOBLE, *J. Chem. Soc.*, (1955) 3106.
23 E. TOMMILA AND C. N. HINSHELWOOD, *J. Chem. Soc.*, (1938) 1806.
24 N. C. CRAIG AND G. B. KISTIAKOWSKY, *J. Am. Chem. Soc.*, 80 (1958) 1574.
25 A. W. LAKE AND G. LOWE, *Biochem. J.*, 101 (1966) 402.