# Ficin- and Papain-Catalyzed Reactions. Changes in Reactivity of the Essential Sulfhydryl Group in the Presence of Substrates and Competitive Inhibitors*

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ABSTRACT: Binding of the substrate, α-*N*-benzoyl-L-arginine ethyl ester (for ficin only), and the competitive inhibitors, α-*N*-benzoyl-D-arginine ethyl ester and *N*-benzoyl β-aminoethanol, in the active site of ficin and of papain enhances the reactivity of the essential sulfhydryl group toward alkylating reagents. Part, but not all, of this influence of the ligand (substrate or inhibitor) is due to bringing a positive charge into the active site. The degree of enhancement of reactivity of the sulfhydryl group is independent of the nature of the alkylating reagent. There is no measurable effect of α-*N*-benzoyl-L-arginine ethyl ester on the reactivity of the essential sulfhydryl group of papain. In the presence of saturating amounts of *N*-benzoylglycine ethyl ester, reaction of the sulfhydryl group of papain and of ficin with alkylating reagents is completely prevented. There is no effect of ligand on the p*K* of the essential sulfhydryl group of ficin. These results are interpreted in terms of (1) deacylation being the rate determining step in the hydrolysis of *N*-benzoylglycine ethyl ester by both ficin and papain, (2) acylation (or a prior step) being rate determining in the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester by ficin, and (3) both acylation (or a prior step) and deacylation being rate determining in the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester by papain ($k_{(acylation)}/k_{(deacylation)} \sim 5$). The possibility that a conformational change is induced in the active site of the enzyme on binding of substrate is considered.

Ficin and papain are plant proteolytic enzymes which have an essential sulfhydryl group in the active site (Smith and Kimmel, 1960). The sequence of amino acids around this essential sulfhydryl group is similar for papain and ficin (Light *et al.*, 1964; Wong and Liener, 1964). Several investigators have concluded that papain catalyzes hydrolytic reactions by the mechanism shown in equation 1 (Smith, 1958; Whitaker and Bender, 1965; Lowe and Williams, 1965a; Kirsch and Igelström, 1966; Bender and Brubacher, 1966)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{ES'} \xrightarrow[H_2O]{k_3} E + P_2 \qquad (1)$$

where E is enzyme, S is substrate, ES is enzyme–substrate complex, ES′ is the acyl-enzyme intermediate and $P_1$ and $P_2$ would be the alcohol and acid portions from an ester substrate, respectively. ES′ is formed by reaction of the essential sulfhydryl group of the enzyme with the acyl part of the substrate to give a thiol ester intermediate (Lowe and Williams, 1964, 1965a; Bender and Brubacher, 1964; Brubacher and Bender, 1966). The evidence for ficin-catalyzed hydrolyses proceeding by this mechanism is more tentative (Hammond and Gutfreund, 1959; Lowe and Williams, 1964, 1965a; Hollaway, 1968; Whitaker, 1969; Kramer and Whitaker, 1969).

Based on the observation (1) that $k_{cat}/K_m$(app) *vs.* pH and $k_{cat}$ *vs.* pH profiles were identical, and (2) that $K_m$(app) and $K_I$ for α-*N*-benzoyl-L-arginine ethyl ester and α-*N*-benzoyl-D-arginine ethyl ester were identical, we have postulated that acylation must be the rate-controlling step in ficin-catalyzed hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester (Whitaker, 1969). In the present paper, we present additional data in support of this hypothesis. Data on the rate-limiting steps in the hydrolysis of (1) *N*-benzoylglycine ethyl ester by ficin and papain, and (2) α-*N*-benzoyl-L-arginine ethyl ester by papain are also presented.

## Materials and Methods

*Materials.* Component 5 (formerly designated as component G) of *Ficus glabrata* ficin was purified as described previously (Sgarbieri *et al.*, 1964; Williams and Whitaker, 1969) and was electrophoretically and chromatographically homogeneous. Protein concentration of the solutions was determined at 280 mμ using $\epsilon_{280}$ 54,000 (Williams and Whitaker, 1969) based on a molecular weight of 25,000 (Englund *et al.*, 1968). Papain (Worthington Biochemical Corp., lot PAP 7DB, two-times crystallized) was recrystallized as the mercuripapain derivative (Whitaker and Peréz-Villaseñor, 1968). Protein concentration of papain solutions was determined at 280 mμ using $\epsilon_{280}$ 51,000 (Bender *et al.*, 1966).

α-*N*-Benzoyl-L-arginine ethyl ester hydrochloride (lot S-5047), α-*N*-benzoyl-D-arginine ethyl ester hydrochloride (lot L-1984), and *N*-benzoylglycine ethyl ester (lot S-2236) were from Mann Research Laboratories. 2-Chloroacetamide, 2-chloroacetic acid, cysteine hydrochloride, and Versene were from Eastman. 2-Iodoacetamide and casein (Hammarsten quality) were from Nutritional Biochemical Corp. The iodoacetamide was recrystallized from benzene before use.

*N*-Benzoyl β-aminoethanol was synthesized according to

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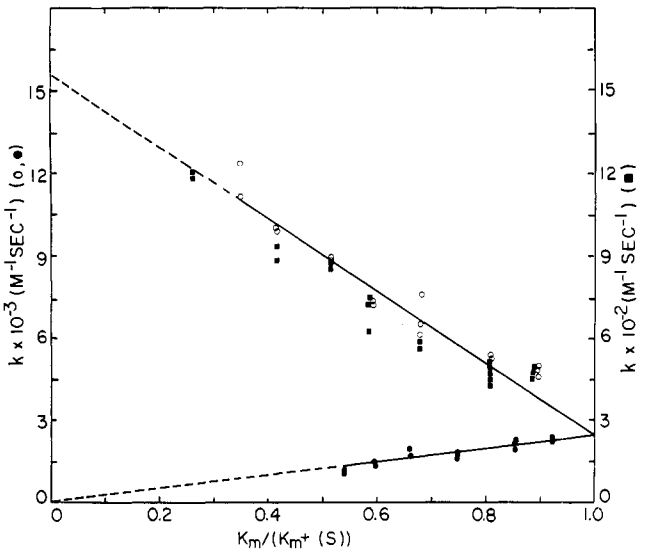


FIGURE 1: Effect of type of substrate and its concentration on the rate of activity loss of ficin in presence of alkylating reagent. Conditions were: pH 6.0, 35.0°, ionic strength of 0.30 and ficin concentration of $3.23 \times 10^{-6}$ M. Chloroacetic acid (■) concentration was $5.00 \times 10^{-4}$ or $1.00 \times 10^{-3}$ M. Iodoacetamide (○,●) concentration was $4.00 \times 10^{-5}$ M. $K_m$(app) values were 0.0425 and 0.0587 M for α-N-benzoyl-L-arginine ethyl ester (○, ■) and *N*-benzoylglycine ethyl ester (●), respectively.

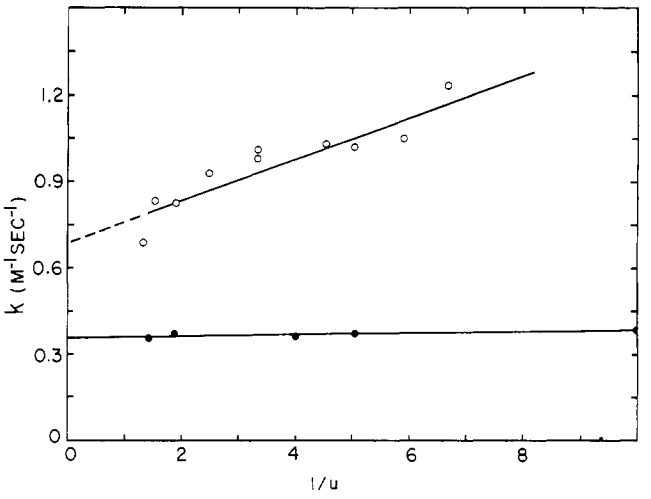


FIGURE 3: Effect of ionic strength on the rate of activity loss of ficin in presence of chloroacetamide and the presence (○) and absence (●) of α-*N*-benzoyl-D-arginine ethyl ester. Conditions were: pH 6.0, 35.0°, chloroacetamide concentration of $4.00 \times 10^{-3}$ M and ficin concentration of $5.80 \times 10^{-6}$ M. The ionic strength was adjusted with potassium chloride.

the method of Fry (1949). The melting point was 61.0–62.0° for the first crop (major) of crystals. The second crop of crystals, with melting point of 63.2–64.2° (lit. (Fry, 1949) mp 61–63°), was used in the work reported here. All other compounds were reagent grade and deionized water was used throughout the experiments.

*Methods.* Rates of inactivation of ficin and papain through reaction with the halo acids and derivatives were determined by two methods. In the first method, as described by Sluyterman (1968), the reactions were performed in a Radiometer titrator (Titrator 11, titragraph SBR 2c, pH meter 25, buret SBU1A) thermostated at 35.0°. The reactions were performed at pH 6.0 in the presence of 0.01 M sodium phosphate, $1.0 \times 10^{-3}$ M Versene, $5.0 \times 10^{-3}$ M cysteine and sufficient potassium chloride to give a final ionic strength of 0.30. The reaction was allowed to proceed in the absence of inhibitor for about 1–2 min in order to obtain the initial rate, then the inhibitor was added. The pseudo-first-order rate constant for the inactivation of the enzyme was calculated from the data as described by Sluyterman (1968). The second-order rate constant was obtained by dividing the pseudo-first-order constant by the inhibitor concentration. Data of Figure 1 were determined by this method.

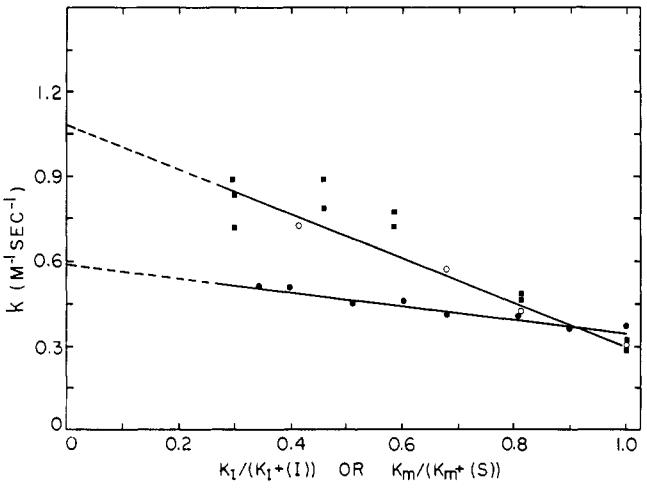


FIGURE 2: Effect of type of ligand and its concentration on the rate of activity loss of ficin in presence of chloroacetamide. Conditions were: pH 6.0, 35.0°, ionic strength of 0.30, chloroacetamide concentration of $4.00 \times 10^{-3}$ M and ficin concentration of $5.80 \times 10^{-6}$ M. $K_I$ values were 0.0426 and 0.208 M for α-*N*-benzoyl-D-arginine ethyl ester (■) and *N*-benzoyl β-aminoethanol (●), respectively; $K_m$(app) value for α-*N*-benzoyl-L-arginine ethyl ester (○) was 0.0425 M.

The second method was designed primarily for determining the effect of competitive inhibitors on the rate of the alkylation reaction. The reactions were performed at 35.0° and pH 6.0 (except those designed to measure the p*K* of the sulfhydryl group). The reactions contained 0.1 M sodium phosphate, $1.0 \times 10^{-3}$ M Versene, $5.0 \times 10^{-3}$ M cysteine, sufficient potassium chloride to give a final ionic strength of 0.30 and the desired amount of ligand. The reaction was initiated by addition of the alkylating reagent. Aliquots were removed at intervals into tubes containing 1% casein, $1.25 \times 10^{-2}$ M cysteine, $1.25 \times 10^{-2}$ M Versene, and 0.1 M sodium phosphate, all at pH 7.0. The activity left was determined at 35.0° according to the method of Kunitz (1947) as modified for ficin assay (Sgarbieri *et al.*, 1964). Pseudo-first-order rate constants were determined from the rates of activity loss and converted into second-order rate constants by dividing by alkylating reagent concentration. At pH 8.0 and above, the reactions followed second-order rate kinetics because of the low alkylating reagent concentrations used. Data of Figures 2–5 were determined by this method.

$K_m$(app) values were determined from the initial reaction rate data before the addition of alkylating reagent. $K_I$ values were determined under the same conditions described above

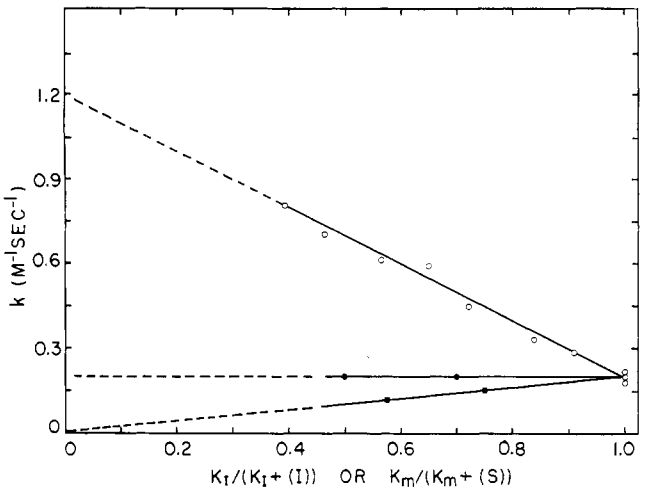


FIGURE 4: Effect of type of ligand and its concentration on the rate of activity loss of papain in presence of chloroacetamide. Conditions were: pH 6.0, 35.0°, ionic strength of 0.30, chloroacetamide concentration of $4.00 \times 10^{-5}$ M and papain concentration of $4.80 \times 10^{-5}$ M. $K_m$(app) values were 0.015 M (Whitaker and Bender, 1965) and 0.021 M (Sluyterman, 1964) for α-*N*-benzoyl-L-arginine ethyl ester (●) and and *N*-benzoylglycine ethyl ester (■), respectively. $K_I$ value of α-*N*-benzoyl-D-arginine ethyl ester (○) was determined to be 0.130 ± 0.022 M.

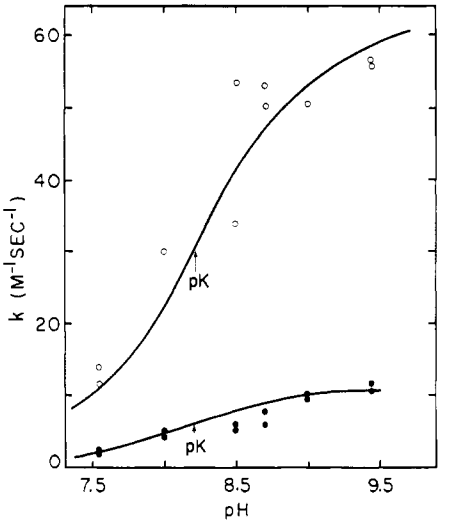


FIGURE 5: Effect of pH on the rate of activity loss of ficin in the presence of chloroacetamide and the presence (○) and absence (●) of α-*N*-benzoyl-D-arginine ethyl ester. Conditions were: 35.0°, ficin concentration of $5.80 \times 10^{-6}$ M, sodium phosphate buffer at pH 7.55, sodium borate buffer at the other pH values, ionic strength of 0.30. Chloroacetamide concentrations ranged from $1.60 \times 10^{-4}$ M at pH 7.55 to $1.78 \times 10^{-5}$ M at pH 9.45. The lines are calculated for a p$K$ of 8.3 and $k_{max}$ of 11.6 and 61.9 $M^{-1}$ $sec^{-1}$ in the absence and presence of 0.1 M α-*N*-benzoyl-D-arginine ethyl ester, respectively. The p$K$ values were determined by plotting the data as log $\frac{E_{act}}{E_{inact}}$ *vs.* pH or $\Delta k/\Delta$pH *vs.* pH.

using 0.005 M α-*N*-benzoyl-L-arginine ethyl ester as the substrate and various concentrations of the competitive inhibitor. The rates of the reactions were followed in a pH-Stat. $K_I$ was calculated from eq 2

$$V_0'/V_0 = [K_m(app)\left(1 + \frac{(I)}{K_I}\right) + (S)/[K_m(app) + (S)] \qquad (2)$$

where $V_0$ and $V_0'$ are the observed rates in the absence and presence of competitive inhibitor, respectively, (I) is the competitive inhibitor concentration, (S) is the substrate concentration and $K_I$ is the dissociation constant of the enzyme–inhibitor complex. $K_m$(app) was taken as 0.015 M (Whitaker and Bender, 1965) for papain acting on α-*N*-benzoyl-L-arginine ethyl ester.

## Results

*Effect of Ligand Concentration on Rate of Inactivation of Ficin.* The effect of substrate concentration on the rate of activity loss of ficin by reaction with alkylating reagents is shown in Figure 1. On the ordinate is plotted the second-order rate constant for loss in activity of the enzyme and on the abscissa is plotted $K_m/[K_m + (S)]$. In the absence of substrate $K_m/[K_m + (S)]$ is 1.0 and in the presence of a large excess of substrate ((S) → ∞) $K_m/[K_m + (S)]$ approaches zero. When the (S) → ∞ for α-*N*-benzoyl-L-arginine ethyl ester the rate of activity loss is some seven times faster than in the absence of substrate. The same results are obtained whether one uses the neutral alkylating reagent, iodoacetamide, or the negatively charged reagent, chloroacetate. While the neutral reagent reacts at a 10-fold faster rate, the increased rate of activity loss in the presence of substrate is not a function of the charge on the alkylating reagent. In contrast to the results obtained when α-*N*-benzoyl-L-arginine ethyl ester is the substrate, the presence of *N*-benzoylglycine ethyl ester at (S) → ∞ prevents the loss of activity due to reaction with the alkylating reagent.

In order to follow conveniently the rate of activity loss by the second method (see Methods section), the less reactive alkylating reagent, chloroacetamide, was used. Over the range of alkylating reagent used ($0.1 \times 10^{-3}$–$1.2 \times 10^{-3}$ M) the rate of activity loss followed pseudo-first-order kinetics. At pH 6.0, 35.0°, ionic strength of 0.30 and ficin concentration of $5.80 \times 10^{-6}$ M, the second-order rate constant was 0.294 $M^{-1}$ $sec^{-1}$. For iodoacetamide and chloroacetic acid, the second-order rate constants, as determined by the pH-Stat method, were $2.50 \times 10^3$ and $2.50 \times 10^2$ $M^{-1}$ $sec^{-1}$, respectively.

As shown by the data in Figure 2, the effects of the substrate, α-*N*-benzoyl-L-arginine ethyl ester and the competitive inhibitor, α-*N*-benzoyl-D-arginine ethyl ester, on the rate of activity loss of ficin in the presence of chloroacetamide are identical. There is a 3.7-fold increase in the rate of activity loss when (S) or (I) → ∞. The fold increase obtained by this method is less than that obtained by the pH-Stat method. Perhaps the kinetic method (pH-Stat) does not adequately take into account the rate of decrease in activity due to depletion of substrate.

The question arises as to what effect the positive charge on the ligands, α-*N*-benzoyl-L-arginine ethyl ester and α-*N*-benzoyl-D-arginine ethyl ester, has on the rate of reaction with the alkylating reagent. To answer this question a neutral competitive inhibitor was needed. A number of neutral compounds were examined but most of them proved to be non-

inhibitory or did not show strictly competitive inhibition. *N*-Benzoyl β-aminoethanol behaved strictly as a competitive inhibitor with a $K_I$ of 0.208 M as compared with a $K_m$(app) of 0.0587 M for *N*-benzoylglycine ethyl ester. The effect of this compound on the rate of reaction of ficin with chloroacetamide is shown in Figure 2. When (I) → ∞ the rate of activity loss of ficin in the presence of chloroacetamide is increased by 2-fold.

The difference between the 2-fold effect exerted by the neutral ligand and the 3.7-fold effect caused by the positively charged ligands must represent the effect of the positive charge on the rate of reaction of ficin with chloroacetamide. To substantiate this the influence of the positive charge was suppressed by increasing the ionic strength of the reaction mixture. These results are shown in Figure 3. There was no effect of ionic strength on either the rate of activity loss due to reaction with chloroacetamide in the absence of ligand or on $K_I$ for α-*N*-benzoyl-D-arginine ethyl ester. In contrast, the effect of the positive ligand, α-*N*-benzoyl-D-arginine ethyl ester, on the rate of activity loss was decreased as the ionic strength was increased. The decrease at infinite ionic strength was essentially that predicted based on the results obtained with *N*-benzoyl β-aminoethanol and α-*N*-benzoyl-D-arginine ethyl ester at an ionic strength of 0.30 (Figure 2).

*Effect of Ligand Concentration on Rate of Inactivation of Papain.* The effects of the concentration of α-*N*-benzoyl-L-arginine ethyl ester, α-*N*-benzoyl-D-arginine ethyl ester and *N*-benzoylglycine ethyl ester on the rate of activity loss of papain on reacting with chloroacetamide are shown in Figure 4. When the concentration of α-*N*-benzoyl-D-arginine ethyl ester approaches infinity, there is a 6-fold increase in the rate of activity loss in the presence of chloroacetamide as compared with the rate of activity loss in the absence of ligand. This is about 1.5 times the effect found with ficin. However, in marked contrast to the effect of α-*N*-benzoyl-D-arginine ethyl ester on papain and the results with ficin, there was no apparent effect of α-*N*-benzoyl-L-arginine ethyl ester on the rate of activity loss of papain in the presence of chloroacetamide. The limited results reported here are in complete agreement with those reported by Sluyterman (1968) as are those results with *N*-benzoyglycine ethyl ester which show that chloroacetamide cannot inhibit papain in the presence of saturating amounts of the substrate. Because of this agreement we did not obtain more extensive data.

*Effect of pH on Activity Loss in Presence and Absence of Ligand.* The enhancement in rate of activity loss in the presence of ligand and alkylating reagent could be explained by a shift downward in the p$K$ of the group reacting with the alkylating reagent. In order to check this possibility the effect of pH on the rate of activity loss was determined in the presence and absence of 0.1 M α-*N*-benzoyl-D-arginine ethyl ester. The results are shown in Figure 5. The marked enhancement in rate of reaction with chloroacetamide in the presence of ligand is apparent from these data. The degree of enhancement (5.4-fold) is exactly identical at all pH values, indicating that the p$K$ of the group reacting with chloroacetamide is not changed by the presence of the ligand. The p$K$ of the group which reacts with chloroacetamide is 8.3 in the presence and absence of ligand. Ignoring for the moment the complete pH–rate profile of Figure 5, one can calculate that it would require a shift of one full p$K$ unit (p$K$ = 7.3) in order to explain the degree of enhancement found. Such a marked shift in p$K$ would easily be detected and does not show up in the data of Figure 5.

## Discussion

From the known reactivities of iodoacetamide, chloroacetamide and chloroacetic acid one would expect, *a priori*, that they would react readily with the essential sulfhydryl groups of papain and ficin to rapidly inactivate these enzymes. Papain has only one sulfhydryl group (Light *et al.*, 1964) while ficin has two (Liener, 1961). However, the second sulfhydryl group in ficin is probably buried since it reacts with alkylating reagents only after denaturation (Liener, 1961). Previously, it has been shown that the rate of loss of activity of ficin and papain in the presence of alkylating reagents corresponds exactly to the rate of loss of the essential sulfhydryl groups of these enzymes (Holloway *et al.*, 1964; Sluyterman, 1968). The data presented in this paper on the effect of pH on the rate of inactivation of ficin with chloroacetamide (Figure 5) also indicate the group which is alkylated is probably a sulfhydryl group (p$K$ = 8.3). This p$K$ of 8.3 is in agreement with that found previously for ficin reacting with chloroacetamide and with iodoacetamide (Holloway *et al.*, 1964) and with papain reacting with L-1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK; Bender and Brubacher, 1966; Whitaker and Peŕez-Villaseñor, 1968).

At pH 6.0 and 35.0°, the second-order rate constants for reaction of iodoacetamide, chloroacetic acid, and chloroacetamide with the essential sulfhydryl group of ficin are $2.50 \times 10^3$, $2.50 \times 10^2$, and 0.294 $M^{-1}$ $sec^{-1}$, respectively. The rate of reaction with iodoacetic acid was too rapid at pH 6.0 to be measured under pseudo-first-order rate conditions. It is to be expected that iodoacetamide would react at a faster rate with a sulfhydryl anion than would chloroacetamide. However, the 850-fold greater reactivity of chloroacetic acid as compared with chloroacetamide is totally unexpected. In most cases, the amide is found to be more reactive than the corresponding acid (Webb, 1966) as would be expected based on the relative electron withdrawing and resonance capabilities of the amide and carboxylate groupings. The essential sulfhydryl group of papain was found to react with chloroacetic acid some 30,000 times faster than does the sulfhydryl group of cysteine (Sluyterman, 1968). The marked reactivity of the chloroacetic acid with the essential sulfhydryl group is even more surprising since negatively charged compounds bind very poorly with papain and ficin (Stockell and Smith, 1957; Smith *et al.*, 1958; Sluyterman, 1964; Whitaker and Bender, 1965; Whitaker, 1969). For example, the $K_I$ the α-*N*-benzoyl-L-arginine increases 7- to 20-fold when the carboxyl group is ionized (Sluyterman, 1964; Whitaker and Bender, 1965). Previously, we have suggested that, even though it does not appear to be involved in binding the substrate, there seems to be a negatively charged grouping in or near the active site of papain (Whitaker, 1969).

The presence of α-*N*-benzoyl-L-arginine ethyl ester (ficin only) and α-*N*-benzoyl-D-arginine ethyl ester in the active site increases some 3- to 7-fold the rate of reaction of the essential sulfhydryl group of ficin and of papain with alkylating reagents. On the other hand, the rate of reaction of the essential sulfhydryl group of ficin and of papain with alkylating reagents is prevented by saturating amounts of *N*-benzoylglycine ethyl ester. In agreement with Sluyterman (1968),

we believe this to be strong evidence for different rate determining steps in the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester *vs.* *N*-benzoylglycine ethyl ester.

The acylation of the essential sulfhydryl group of these enzymes by the substrate in the course of the hydrolysis reaction seems to be sufficiently well documented (Lowe and Williams, 1964, 1965a; Bender and Brubacher, 1964; Whitaker and Bender, 1965; Brubacher and Bender, 1966). If proof of an acyl-enzyme intermediate is accepted then the minimum generalized reaction one could write for these enzymes is given by eq 1.

In those reactions where deacylation, $k_3$, is the totally rate-controlling step, there should be no reaction of the enzyme with alkylating reagents in the presence of saturating amounts of substrate since all the enzyme will be in the ES′ (thiol ester) form. One could also postulate that the rate of reaction of the essential sulfhydryl group might be decreased if there were a steric effect of the substrate bound to the enzyme. The data presented here do not support this concept. As shown by the theoretical treatment in Figure 6, regardless of the relative rates of reaction of E and ES with alkylating reagent, the same results will be obtained in all cases where deacylation, $k_3$, is the rate-limiting step (line A in all cases). Therefore, it appears that deacylation is the rate-controlling step for the hydrolysis of *N*-benzoylglycine ethyl ester by both ficin and papain. This is supported by other data for papain (Lowe and Williams, 1965c; Kirsch and Igelström, 1966; Henry and Kirsch, 1967) and ficin (Hollaway, 1968).

For those reactions where acylation (or a prior step) is the rate-determining step the essential sulfhydryl group should be free and capable of reacting with an alkylating reagent at the same rate in the presence or absence of substrate (provided there is no perturbing influence of the substrate). In the case of ficin, the reactivity of the essential sulfhydryl group actually increases in the presence of substrate. We shall speculate as to the reasons for this later. The data showing the marked enhancement in reactivity of the essential sulfhydryl group of ficin in the presence of α-*N*-benzoyl-L-arginine ethyl ester would rule out the possibility that deacylation is rate determining for the hydrolysis of this substrate. Rather, it would appear that the acylation step (controlled by $k_2$), or some step prior to the acylation step, is rate controlling. This is further supported by the data on $K_m$(app) and $K_I$ for α-*N*-benzoyl-L-arginine ethyl ester and α-*N*-benzoyl-D-arginine ethyl ester which were found to be 0.0425 and 0.0426 M, respectively. From eq 1 it may be shown that $K_m$(app) is given by eq 3.

$$K_m(\text{app}) = \frac{k_3(k_{-1} + k_2)}{k_1(k_2 + k_3)} \qquad (3)$$

If $k_2$ is much smaller than $k_{-1}$ and $k_3$ then $K_m$(app) = $k_{-1}/k_1$. The effects of α-*N*-benzoyl-L-arginine ethyl ester and α-*N*-benzoyl-D-arginine ethyl ester on the reactivity of the essential sulfhydryl group of ficin were identical (Figure 2) again indicating that $K_m$(app) = $K_s$ and thus $k_2 < k_{-1}$ and $k_3$. The same conclusion has been reached in an investigation of the influence of pH on $K_m$(app) and $K_I$ for ficin (Whitaker, 1969) and the effect of proflavin on the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester by ficin (Hollaway, 1968). As shown by the theoretical treatment in Figure 6 only when $k_{ES}/k_E > 1$ and $k_2 \lesssim k_3$ (case IV, lines B and C) can one expect to see an increase in reactivity of the essential sulfhydryl group.

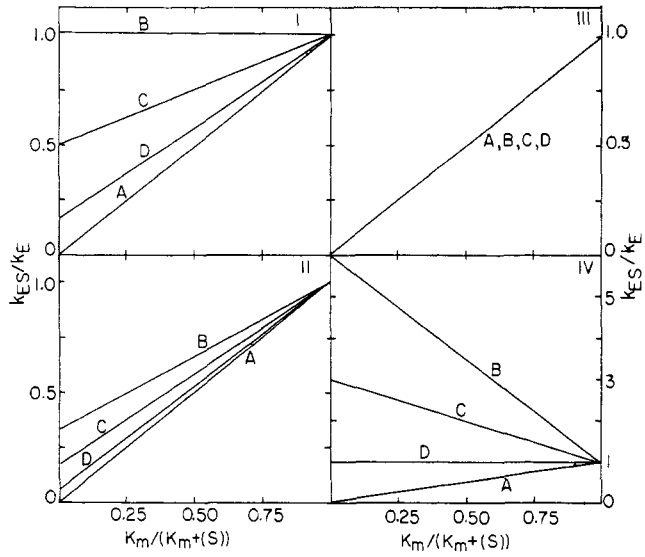


FIGURE 6: Theoretical calculations of interrelationships among ligand concentration, relative rates of reaction of *E*, $k_E$, and ES, $k_{ES}$, with alkylating reagent, and acylation rate, $k_2$, and deacylation rate, $k_3$. The following relationships were used: $[E_t] = [E] + [ES] + [ES']$; $[E] = [E_t]K_m/[K_m + [S]]$; $[ES']/[ES] = k_2/k_3$. Four cases considered were: I, $k_{ES}/k_E = 1.0$; II, $k_{ES}/k_E = 0.333$; III, $k_{ES}/k_E = 0$; IV, $k_{ES}/k_E = 6.0$. Four situations considered in each case were: A, $k_2 \gg k_3$; B, $k_2 \ll k_3$; C, $k_2 = k_3$; D, $k_2 = 5k_3$.

While the effect of *N*-benzoylglycine ethyl ester on the reactivity of the essential sulfhydryl group of ficin and of papain was the same (compare Figures 1 and 4) there was a marked difference between ficin and papain in the effect of α-*N*-benzoyl-L-arginine ethyl ester on the reactivity of the essential sulfhydryl group. In the case of papain, there appears to be no effect of α-*N*-benzoyl-L-arginine ethyl ester on the reactivity of the essential sulfhydryl group. As shown by the theoretical calculations of Figure 6 only two situations should lead to such an experimental observation. These are (1) where $k_{ES}/k_E = 1$ and $k_2 \ll k_3$ (case 1, line B) and (2) where $k_{ES}/k_E > 1$ and $k_2$ and $k_3$ are of the same order of magnitude (case IV, line D). On the other hand, there is a marked increase in the reactivity of the sulfhydryl group of papain in the presence of α-*N*-benzoyl-D-arginine ethyl ester. The data with the competitive inhibitor are not complicated by additional steps (acylation and deacylation steps) in the reaction and should, therefore, reflect the true influence of placing the substrate in the binding site of the enzyme. We believe these apparently incongruous data for substrate and competitive inhibitor mean that both the acylation step and the deacylation step are rate controlling in the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester by papain; in other words, $k_2$ and $k_3$ are of the same order of magnitude.

Based on the effect of saturating amounts of α-*N*-benzoyl-D-arginine ethyl ester on the reactivity of the essential sulfhydryl group of papain ($k_{ES}/k_E = 6.0$, Figure 4) and on the postulate that the effect of α-*N*-benzoyl-L-arginine ethyl ester and α-*N*-benzoyl-D-arginine ethyl ester on the reactivity of the essential sulfhydryl group should be the same or similar we have calculated $k_2/k_3$. This calculation shows $k_2 = 5.0k_3$. Line D (case IV) of Figure 6 is drawn on the basis of $k_{ES}/k_E$

= 6.0 and $k_2/k_3$ = 5.0. As indicated, there is no observed effect of various concentrations of α-*N*-benzoyl-L-arginine ethyl ester on the rate of activity loss. $k_2$(lim) and $k_3$(lim) for the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester by papain were determined by Whitaker and Bender (1965) to be 64.9 ± 13.9 and 20.2 ± 1.7 $sec^{-1}$, respectively. The ratio of $k_2$(lim)/$k_3$(lim) of 3.2 ± 1.0 is in reasonable agreement with the ratio of $k_2/k_3$ of 5.0 calculated by the above method.

Let us now turn to the enhanced reactivity of the essential sulfhydryl group of ficin and of papain with alkylating reagents in the presence of ligands. The reactivity of the sulfhydryl anion could be increased either by making the group a stronger acid or by making it more accessible to the alkylating reagent. In the first case, binding of the ligand to the active site could disrupt binding of the hydrogen of the sulfhydryl group with another group on the enzyme (increase in p*K*, reduced reactivity at constant pH) or could supply a site for hydrogen bonding to the ligand (decrease in p*K*). Data indicating that the p*K* of the sulfhydryl group does not change in the presence of ligand (Figure 5) do not support this case.

The alternate possibility that the reactivity of the sulfhydryl group is increased in the presence of ligand because of its increased accessibility to alkylating reagent is an intriguing one. The marked reactivity of the essential sulfhydryl groups of ficin and papain toward alkylating reagents suggests that these compounds may be bound to the enzyme near the sulfhydryl group before reacting. The further increased rate of reaction in the presence of ligand (substrate or competitive inhibitor) could be due to (1) better binding of alkylating reagent to enzyme in the presence of ligand, (2) complex formation between ligand and alkylating reagent followed by binding to enzyme, or (3) a conformational change induced in the active site of the enzyme on binding of ligand which permits the alkylating reagent to react more rapidly.

The possibility of a conformational change in the active site of ficin in the presence of substrate (Holloway *et al.*, 1964) and proflavin (Hollaway, 1968) has been suggested. Carty and Kirschenbaum (1964) reported that hippuric acid enhanced the rate of heat denaturation of nonactivated papain. Perhaps this labilization of enzyme in the presence of hippuric acid is due to a conformational change caused by binding of the ligand. Conformational changes in the active sites of enzymes on binding or transforming of substrates are well documented (Yankeelov and Koshland, 1965; Bernhard *et al.*, 1966; Massey and Curti, 1966; Lui and Cunningham, 1966; Reeke *et al.*, 1967; Sigler *et al.*, 1968; Wright *et al.*, 1969). In one of the largest conformational changes found, a tyrosyl group in carboxypeptidase A moves some 14 Å in the presence of the substrate, glycyl-L-tyrosine (Reeke *et al.*, 1967).

Two of the more pressing needs for understanding the mechanism of action of papain and ficin involve answers to the questions of (1) whether a carboxyl group (Bernhard and Gutfreund, 1956; Stockell and Smith, 1957; Hammond and Gutfreund, 1959; Whitaker and Bender, 1965; Bender and Brubacher, 1966) or an imidazole group (Lowe and Williams, 1965b, c; Husain and Lowe, 1968; Drenth *et al.*, 1968) is involved in the active site and (2) why the substrates α-*N*-benzoyl-L-arginine ethyl ester and α-*N*-benzoyl-L-argininamide are hydrolyzed at essentially the same rates since the acylation step appears to be the rate-determining step for hydrolysis of the ester. Perhaps answers to both these questions will be known when one can answer the question about a possible conformational change on binding of substrate and the rate of this change. We are actively pursuing this problem.

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# The Estimation of Molecular Weights in Mixtures of Two Proteins by the Meniscus Depletion Method*

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ABSTRACT: Sedimentation equilibrium experiments with mixtures of two proteins in different proportions have been performed at speeds high enough to deplete effectively the meniscus region of protein in an attempt to establish what conditions are necessary before the molecular weights and proportions of two proteins can be estimated with reasonable accuracy using this technique. The mixtures used were 20% ovalbumin plus plasma albumin, 5, 10, and 20% ribonuclease plus ovalbumin and 5, 10, and 20% ribonuclease plus plasma albumin. Molecular weights were evaluated from the slopes of $\ln j$ *vs.* $r^2$ plots, from extrapolation of reciprocal plots of $M_w$ and $M_n$ to infinite dilution and from "two species plots."

The possibility of estimating the proportions of the two proteins present in the original solution by extrapolating $M_n$ to the cell bottom was investigated. Experiments with the individual proteins are also reported. It is concluded that if $\sigma$ ($\sigma = \omega^2 M(1 - v\rho)/RT$) $\simeq 5$ for the species of lower molecular weight, the two species plot gives values for the molecular weight, $M_1$, of this species to better than 7% for the ribonuclease–plasma albumin mixtures ($M_2/M_1 = 5$) and within about 10–15% for the ribonuclease–ovalbumin mixtures ($M_2/M_1 = 3.4$) and is considerably better than the other methods tried for evaluating $M_1$. $M_2$ was obtained to better than 7% accuracy for all combinations above 5% ribonuclease–ovalbumin by using the two species plot. Attempts at estimating the proportions of the two species present were unsuccessful even in the mixture of 20% ribonuclease and plasma albumin because of the large errors in $M_n$ in solutions of low concentrations. It is thought that these errors were probably due to adsorption of protein on the walls of the centerpiece.

The method of measuring molecular weights at sedimentation equilibrium by running the ultracentrifuge at such high speeds that a region of the solution near the meniscus becomes effectively free of macromolecules was developed by Yphantis (1964) and has since been used widely. The main attractions of the method, referred to here as the meniscus depletion method, are the moderate times necessary to reach sedimentation equilibrium, the small amount of protein required, the fact that the initial concentration of the solution need not be measured and the increased sensitivity of detection of low molecular weight proteins in paucidiperse solutions which results from the centrifugal fractionation. An experimental investigation of the last point with mixtures of two proteins is the particular concern of the present paper.

In his paper Yphantis (1964) discusses thoroughly the pitfalls of the method and gives some general guide lines based on calculated distributions for the detection of the presence of smaller species for various combinations of molecular weight ratios and concentrations. We thought it would be useful to examine some actual mixtures of two proteins containing small amounts of the lower molecular weight protein to see under what conditions heterogeneity could be detected and how accurately the molecular weight of the smaller protein could be measured. Such information is of immediate interest in this laboratory in the investigation of wool and muscle proteins which are extremely difficult to purify completely and in which a small degree of molecular weight heterogeneity seems likely to persist even after repeated fractionation. After this work had been started, Roark and Yphantis

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