Braun, V., and Schroeder, W. A. (1967), *Arch. Biochem. Biophys. 118*, 241.
Brewer, J. M., and Ashworth, R. B. (1969), *J. Chem. Educ. 46*, 41.
Chervenka, C. H. (1970), *Anal. Biochem. 34*, 24.
Figarella, C., Clemente, F., and Guy, O. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3*, 351.
Folk, J. E., and Schirmer, E. W. (1965), *J. Biol. Chem. 240*, 181.
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., and Singh, R. M. M. (1966), *Biochemistry 5*, 467.
Gratecos, D., Guy, O., Rovery, M., and Desnuelle, P. (1969), *Biochim. Biophys. Acta 175*, 82.
Hartley, B. S. (1964), *Nature (London) 201*, 1284.
Hartley, B. S., and Kauffman, D. L. (1966), *Biochem. J. 101*, 229.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Keller, P. J., and Allan, B. J. (1967), *J. Biol. Chem. 242*, 281.
Matsubara, H., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 175.
Nazarian, G. M. (1968), *Anal. Chem. 40*, 1766.
Petra, P. H., Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1969), *Biochemistry 8*, 2762.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.
Schachman, H. K. (1967), Ultracentrifugation in Biochemistry, New York, N. Y., p 82.
Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.
Schyns, R., Bricteux-Gregoire, S., and Florkin, M. (1968), *Biochim. Biophys. Acta 175*, 97.
Smillie, L. B., Furka, A., Nagabhushan, N., Stevenson, K. J., and Parkes, C. O. (1968), *Nature (London) 218*, 343.
Smillie, L. B., and Hartley, B. S. (1967), *Biochem. J. 105*, 1125.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Travis, J. (1968), *Biochem. Biophys. Res. Commun. 30*, 730.
Travis, J., and Roberts, R. C. (1969), *Biochemistry 8*, 2884.
Warburg, O., and Christian, W. (1942), *Biochem. Z. 310*, 384.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Demonstration of a Change in the Rate-Determining Step in Papain- and Ficin-Catalyzed Acyl-Transfer Reactions*

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ABSTRACT: Advantage was taken of the sensitivity of the rates of deacylation of acyl-papains and ficins to increases effected by added nucleophiles in order to change the rate-determining steps in the reactions of several esters from deacylation to acylation and thereby permit the direct study of the acylation step under steady-state conditions. The change in the rate-limiting step was demonstrated by measuring the initial rates of release of alcohol from ester substrates in the presence of increasing concentrations of amine. The observed velocities increased to a plateau level as the concentration of amine was raised. At the plateau level the rate constant for deacylation of the acyl-enzyme intermediate exceeds that for acyl-enzyme formation. The plateau velocity was independent of the nucleophile used to change the rate-determining step and it was dependent on a group with an apparent pK_a of 8.65, as expected for rate-determining acylation. At high concentrations of amine where the acylation reaction is rate determining, the constants derived from the Michaelis–Menten equation under steady-state conditions are the rate constant for acylation and the enzyme–substrate dissociation constant. These parameters were obtained for the acylation of papain and ficin by *p*-nitrophenyl esters of hippuric acid and carbobenzoxyglycine; the apparent dissociation constants of the enzyme–substrates complexes were between 0.2 and 0.9 mM. In contrast to the results obtained with the *p*-nitrophenyl ester of carbobenzoxyglycine, the dissociation constant of the complex between papain and the *o*-nitrophenyl ester was immeasurably high (>6 mM). The present data together with those in the literature suggest that the principal mode for the binding of *p*-nitrophenyl esters of *N*-acylamino acids to papain and ficin is nonproductive.

The evidence that the papain-catalyzed hydrolysis of esters proceeds through the two-step mechanism involving an intermediate acyl-enzyme is substantial and has been discussed extensively (*e.g.*, Lowe, 1970). A minimal mechanism is shown in Scheme I, where K_s is the dissociation constant of the enzyme–substrate complex, k_2 the acylation rate constant, and k_3' ($= k_3[H_2O]$) the rate constant for the reaction of the acyl-enzyme with water. For most esters so far examined the rate-determining step has been shown to be deacylation of the acyl-enzyme, *i.e.*, $k_2 \gg k_3'$ (Henry and Kirsch, 1967; Fink and Bender, 1969). The exceptions are α-*N*-benzoylarginine ethyl ester for which the acylation rate is only about five times that of deacylation (Whitaker, 1969), isopropyl hippurate for which the rate-determining step is probably acylation (Lucas and Williams, 1969), and *p*-nitrophenyl hippurate for which it has been suggested that there is a kinetically significant step

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SCHEME I

$$E + S \overset{K_s}{\rightleftharpoons} E\cdot S \xrightarrow{k_2} A\text{-}E \xrightarrow{k_3'} E + P_2$$
$$\qquad\qquad\qquad\qquad\quad + P_1$$

prior to the deacylation reaction, possibly the departure of the leaving *p*-nitrophenol (Henry and Kirsch, 1967).

The acylation reaction depends on two ionizable groups on the enzyme, one of $pK_a = 3$–5 and one of pK_a about 8.5, probably representing either an imidazole or carboxyl functional group and the active-site thiol, respectively. The deacylation reaction, however, depends (from pH 3 to 9.5) only on the former ionization because the thiol group is acylated and therefore not available for titration (Hinkle and Kirsch, 1970, and references therein). Experimentally these factors are manifested in the pH profile for K_m, which for esters is a measure of both the acylation and deacylation reactions and is bell shaped, and the pH *vs.* k_{cat} profile, which reflects only the deacylation and is sigmoid. These considerations suggest, therefore, that at increasing values of pH where acylation rates are decreasing and deacylation rates remain constant, it might be possible with suitably chosen substrates to demonstrate a change in the rate-determining step from deacylation to acylation. A second vehicle for effecting a change in the rate-determining step arises from the fact that the total rate of deacylation is greatly increased by added nucleophiles (Brubacher and Bender, 1966, 1967). In the presence of an added nucleophilic acceptor an acyl-enzyme may react with either the nucleophile (N) or water and the minimal reaction mechanism is seen in Scheme II. At sufficiently high concentrations of

SCHEME II

$$E + S \overset{K_s}{\rightleftharpoons} E\cdot S \xrightarrow{k_2} A\text{-}E \xrightarrow{k_3'} E + P_2$$
$$\qquad\qquad\qquad\qquad\quad + \;|\; k_4[N]$$
$$\qquad\qquad\qquad\qquad\quad P_1 \longrightarrow E + P_3$$

nucleophile the rate constants for deacylation would be expected to exceed those of acylation even at intermediate values of pH, and the latter reaction will therefore become the rate-determining step. Experimentally this would become evident in the leveling off of a plot of initial velocity *vs.* nucleophile concentration. At high concentrations of nucleophile where the acylation reaction is rate determining it should be possible to obtain values of k_2 and K_s under steady-state conditions. It has previously been possible to measure these parameters for active ester substrates only with a stopped-flow apparatus.

It is the purpose of this paper to report the demonstration of a change in rate-determining step from deacylation to acylation in the presence of high concentrations of nucleophiles for the papain-catalyzed reactions of several esters of benzyloxycarbonylglycine and the papain- and ficin-catalyzed reactions of *p*-nitrophenyl hippurate.

Experimental Section

Materials

Tetrathionate-treated papain was prepared as previously described (Hinkle and Kirsch, 1970). Ficin was obtained from Sigma (lot 119B-4750). Tetrathionate-treated ficin (Englund *et al.*, 1968) was prepared as follows. The commercial suspension of ficin (0.5 ml, 12.5 mg) was centrifuged and dissolved in 2 ml of 0.02 M sodium acetate buffer (pH 4.8), containing 0.5 mM EDTA, and 13 mg of sodium tetrathionate (Eastman) was added. After 15 min at room temperature the solution was applied to a 1.5 × 22 cm Bio-Gel P60 column and eluted with 0.02 M sodium acetate buffer (pH 4.8), containing 0.5 mM EDTA at a flow rate of 0.6 ml/min. Fractions containing the major portion of the protein peak (6.6 ml, 4.1 mg) were pooled and stored at 4°. This preparation of tetrathionate-treated ficin was used for all experiments.

Esters of ZGly[1] and pNPH were available from previous studies (Kirsch and Igelström, 1966; Henry and Kirsch, 1967). L-Tryptophanamide·HCl was obtained from Miles Laboratories or Sigma, glycinamide·HCl from Aldrich, and L-histidine methyl ester·$(HCl)_2$ from Sigma. L-Cysteine·HCl was from Nutritional Biochemical Corp.

Acetonitrile was distilled and stored over molecular sieves. Distilled water was used throughout. All inorganic salts and buffers were reagent grade.

Methods

Papain was activated for 15–45 min at room temperature in solutions containing 0.05 M potassium phosphate buffer (pH 6.8), 0.5 mM EDTA, 10–40 mM L-cysteine, and 0.05–0.1 mM tetrathionate-treated papain. Tetrathionate-treated ficin was activated for 90 min in the same buffer containing 3 mM L-cysteine and 40 μM ficin. Solutions of active papain and ficin were diluted in the same buffer, without cysteine, to give final concentrations of 1–30 μM enzyme. Concentrations of ficin were determined spectrophotometrically based on $\epsilon_{280} = 46{,}000$ (Hollaway *et al.*, 1969). The concentration of active papain was determined from its activity toward ZGly*p*NP and enzyme concentrations were standardized to a specific activity of 1.46 (Klein and Kirsch, 1969). This activity is equivalent to an initial velocity of 3.4 μM min^{-1} for a solution at 25°, pH 6.8, containing 0.05 M potassium phosphate buffer, 0.5 mM EDTA, 6.7% acetonitrile, 0.1 mM ZGly*p*NP, and 10 nM papain based on a molecular weight of 23,000 (Drenth *et al.*, 1968).

Amine hydrochlorides were stored at 4° in water and immediately before use were neutralized to the pH of the experiment with KOH, made to 5% CH_3CN, and diluted as necessary in the appropriate buffer. The buffers were 0.0475 M containing 0.475 mM EDTA, 5% CH_3CN, and made to ionic strength 0.475 with KCl. The buffers used were: pH 6.5–7.9, potassium phosphate; pH 8.0–8.5, sodium pyrophosphate; pH 8.6–9.5, sodium borate. Cuvets containing 0.95 ml of buffer + amine were equilibrated at 25.0°. Enzyme (10 μl) was added and reactions started by the addition of substrate in 50 μl of CH_3CN. Final concentrations were: 9.75% CH_3CN, 0.022–0.045 M buffer, 0.22–0.45 mM EDTA, 0–0.5 M amine, 0.033–0.4 mM substrate, 0.01–0.3 μM papain or ficin, and a final ionic strength of 0.4–0.45, except in the one experiment with ZGlyPE and glycinamide when the final ionic strengths covered the range 0.45–0.72. The rate of liberation of the phenol was monitored spectrophotometrically on a Unicam SP 800A or Gilford Model 220 recording spectrophotometer at: 400 mμ (ZGly*p*NP, *p*NPH), 410 mμ

[1] The abbreviations used are: *p*NPH, *p*-nitrophenyl hippurate; $TrpNH_2$, L-tryptophanamide; ZGly, benzyloxycarbonylglycine; *m*NP, *m*-nitrophenyl ester; *o*NP, *o*-nitrophenyl ester; *p*NP, *p*-nitrophenyl ester; PE, phenyl ester.

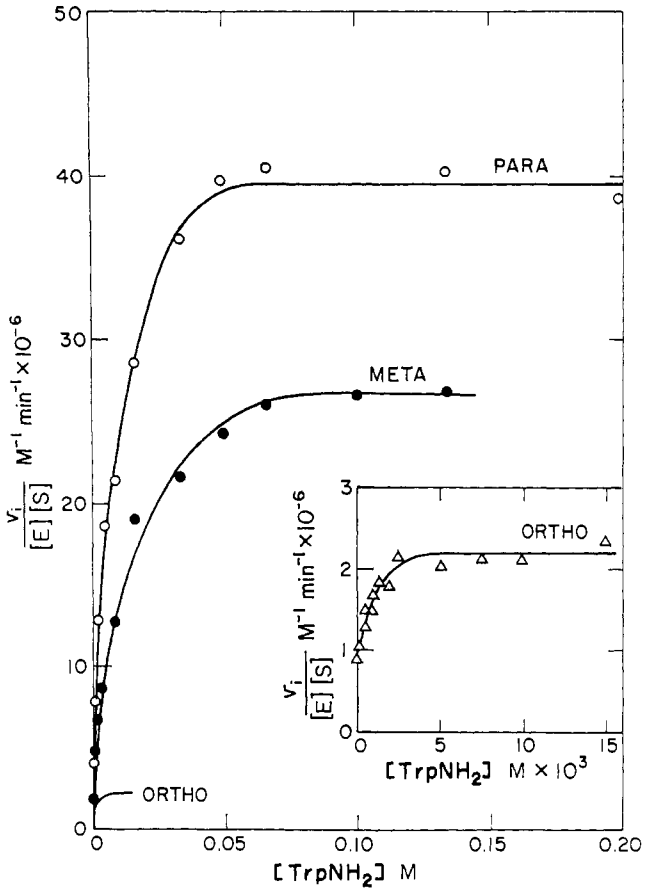


FIGURE 1: The effect of $TrpNH_2$ on the rate of release of *o*-, *m*-, or *p*-nitrophenol from the corresponding nitrophenyl esters of ZGly. The conditions were: 25°, pH 8.25, 9.75% acetonitrile, 0.021–0.045 M sodium pyrophosphate buffer, 0.21–0.45 mM EDTA, and ionic strength 0.4–0.45. Substrate concentrations were: ZGly*p*NP, 0.075 mM; ZGly*o*NP and ZGly*m*NP, 0.33 mM.

(ZGly*o*NP), 330 mμ (ZGly*m*NP), or 270 mμ (ZGlyPE). Initial velocities were obtained from less than the first 5% of the reaction. Nonenzymatic rates were determined separately for all concentrations of substrate and amine under identical conditions and appropriate corrections made. In all cases the nonenzymatic rates were less than 30% of the enzyme-catalyzed rates. Michaelis–Menten parameters were calculated using program HYPERB (Hanson *et al.*, 1967).

Results

Demonstration of a Change in the Rate-Determining Step. The rates of reaction of ZGly nitrophenyl esters were examined in the presence of $TrpNH_2$, a highly reactive nucleophile for *S*-acyl-papains (Brubacher and Bender, 1966; Fink and Bender, 1969). The effect of $TrpNH_2$ concentration on the initial rate of release of nitrophenol from these esters is shown in Figure 1. The observed rate constants increase as the concentration of amine is raised and reach a plateau level where the rates are insensitive to further increases in $TrpNH_2$ concentration. Both the limiting rate constant attained and the dependence of the observed velocities on $TrpNH_2$ concentration were different for the three substrates.

There are, *a priori*, two reasons why the observed rate constants might become independent of nucleophile concentration: (1) the rate-determining step may change from deacylation to acylation; or (2) the acyl-enzymes may become saturated by the nucleophile. The latter possibility is unlikely because the limiting rate constant obtained with a particular substrate is independent of the nucleophile which is used. The reactions of ZGlyPE were examined in the presence of L-histidine methyl ester and glycinamide. As shown in Figure 2 the observed rate constants again increase to a maximum level in the presence of increasing concentrations of nucleophilic amine, and the limiting rate constant is the same with either L-histidine methyl ester or glycinamide. These results, in addition to the pH dependence of the limiting velocities (see below), constitute evidence that the observed rates of disappearance of ZGly ester substrates become independent of nucleophile concentration as a result of a change in rate-limiting step and not because the acyl-enzymes become saturated by the nucleophile.

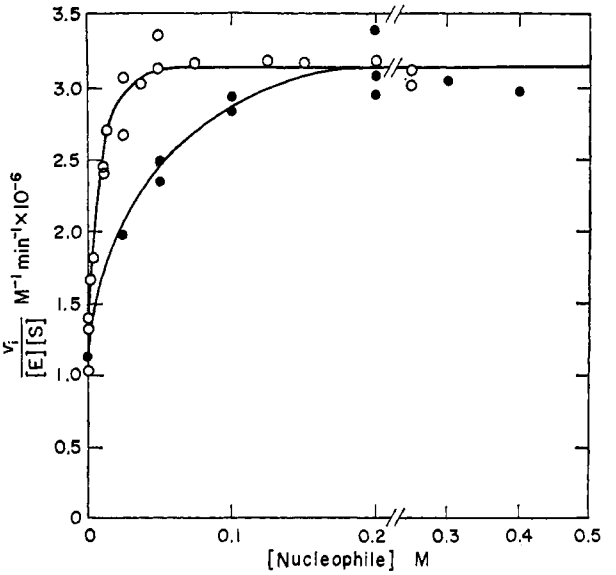


FIGURE 2: The effect of nucleophiles on the rate of release of phenol from ZGlyPE. (○) L-Histidine methyl ester; (●) glycinamide. Conditions were as in Figure 1 except that the ionic strength covered the range 0.45–0.72 in the case of glycinamide. ZGlyPE was 0.15 or 0.187 mM.

The minimal scheme (II) presented for the papain-catalyzed reactions of esters in the presence of nucleophiles leads to the following expression (eq 1) for the initial rate of disappearance of ester at any concentration of substrate [S] and nucleophile [N]. In experiments of the type shown in Figures 1 and

$$\frac{v_i}{[E]} = \frac{k_2(k_3' + k_4[N])[S]}{(k_3' + k_4[N])K_s + (k_2 + k_3' + k_4[N])[S]} \quad (1)$$

2 the rate (v_i/[E]) becomes independent of nucleophile concentration when the rate-determining step has changed from deacylation to acylation, *i.e.*, when $(k_3' + k_4[N]) \gg k_2[S]/(K_s + [S])$ and eq 1 reduces to

$$\frac{v_{lim}}{[E]} = \frac{k_2[S]}{K_s + [S]} \quad (2)$$

Thus the observed rates on the plateau regions of the curves in Figures 1 and 2 (v_{lim}) are a measure of the overall rate of acyl-enzyme formation and are expected to differ with the various ZGly esters, as observed.

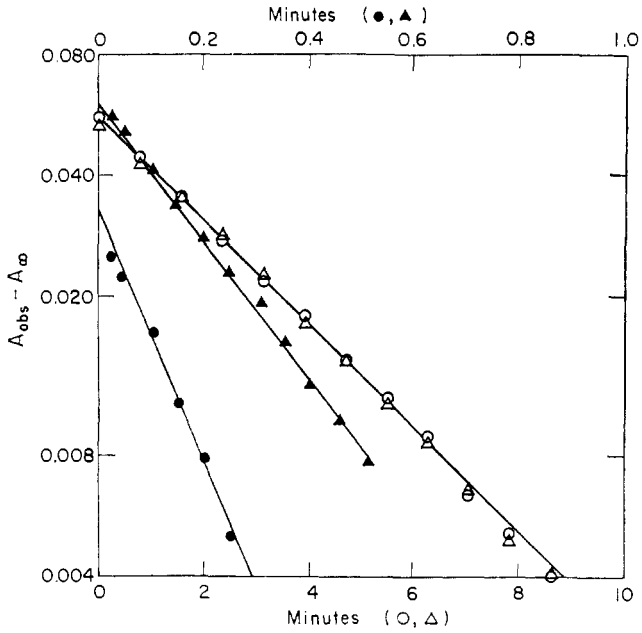


FIGURE 3: First-order plots of the rate of release of *o*- or *p*-nitrophenol from the corresponding esters of ZGly in the presence and absence of $TrpNH_2$. Open symbols are for ZGly*o*NP + (○) no amine (pH 8.05); (△) 10 mM ($TrpNH_2$ (pH 8.10). ZGly*o*NP was 16.7 μM and papain was 0.117 μM. The nonenzymatic rates of hydrolysis of ZGly*o*NP were negligible. Filled symbols are for ZGly*p*NP + (▲) no amine; (●) 95 mM $TrpNH_2$, both at pH 8.27. The ordinate for ZGly*p*NP + $TrpNH_2$ is × 0.1. ZGly*p*NP was either 3.7 μM (no amine) or 30 μM (+ $TrpNH_2$) and papain was 0.142 μM. The rate constants for the nonenzymatic rates of hydrolysis of ZGly*p*NP were 0.018 min^{-1} (no amine) and 0.10 min^{-1} (+ $TrpNH_2$). The conditions for both esters were: 25°, 9.75% acetonitrile, 0.045 M sodium pyrophosphate buffer, 0.45 mM EDTA, and ionic strength 0.45.

Values of k_2/K_s may be obtained by measuring the first-order rate of disappearance of substrate at low concentrations of substrate where $(k_2 + k_3' + k_4[N])[S] \ll (k_3' + k_4[N])K_s$ and eq 1 reduces to

$$\frac{-d[S]}{dt} = \frac{k_2[S][E]}{K_s} \tag{3}$$

or

$$\frac{k_{obsd}}{[E]} = \frac{k_2}{K_s}$$

where k_{obsd} is the first-order rate constant obtained at low concentrations of substrate. The equality

$$\frac{k_{cat}}{K_m} = \frac{k_2}{K_s} \tag{4}$$

where k_{cat} is the measured maximal velocity divided by enzyme concentration and K_m the measured Michaelis constant, will be valid at any concentration of nucleophile according to Scheme II. First-order rate constants were obtained at $[S] \ll K_m$ for the nitrophenyl esters of ZGly in the presence and absence of $TrpNH_2$. The results are shown in Figure 3 and Table I. It can be seen that the ratio $k_2/K_s(k_{cat}/K_m)$ is not affected by the low concentration (5 mM) of $TrpNH_2$ necessary to change the rate-determining step with ZGly*o*NP, but with

TABLE I: Kinetic Parameters for the Reactions of ZGly Esters in the Presence and Absence of Nucleophiles at pH 8.25.

Ester ZGly	[$TrpNH_2$] (mM)	M^{-1} sec^{-1} × 10^{-4} k_{obsd}[a]/[E]	M^{-1} sec^{-1} × 10^{-4} v_{lim}[b]/[E][S]
PE			5.23
*o*NP			3.66
*o*NP		4.34	
*o*NP	5	4.34	
*m*NP			43.4
*m*NP		23.7	
*m*NP	100	56.1	
*p*NP			68.2
*p*NP		59.4	
*p*NP	100	120.0	

[a] Values of $k_{obsd}/[E]$ were obtained from first-order plots at $[S] \ll K_m$ where, from eq 3 and 4: $k_{obsd}/[E] = k_2/K_s = k_{cat}/K_m$. The values given are the average of three or more determinations. Conditions were as in Figure 1 except that substrate concentrations were less than K_m. [b] Values of v_{lim} are the limiting velocities obtained at the plateau regions in Figures 1 and 2 where the initial rates are independent of nucleophile concentration. From eq 2: $v_{lim}/[E][S] = k_2/(K_s + [S])$.

ZGly*m*NP and ZGly*p*NP as substrates the higher concentrations (100 mM) of $TrpNH_2$ used to change to rate-limiting acylation (Figure 1) cause the ratio k_{cat}/K_m to increase. Added nucleophiles have been shown to affect k_{cat}/K_m in other cases (Henry and Kirsch, 1967; Fink and Bender, 1969) and the increases observed in the present case with ZGly*p*NP are similar in magnitude to those observed by Fink and Bender (1969) who used $TrpNH_2$ with *N*-Ac-L-Trp*p*NP as substrate. This finding is not consistent with the simple mechanism (Scheme II) for papain action (see Discussion). In Table I values of k_2/K_s in the presence and absence of nucleophiles are also compared to values of $v_{lim}/([E][S])$ from the plateau regions of Figures 1 and 2. From eq 2, if $K_s \gg [S]$ then $v_{lim}/([E][S]) = k_2/K_s$. Since these values are in only fair agreement, it appears that this condition is not met. However, the fact that the limiting rates obtained at high concentrations of nucleophiles are close to the overall rates of acylation measured under first order conditions with all four ZGly esters is supportive evidence for a change in the rate-determining step.

pH Dependence of the Reactions of ZGlyoNP. Values of k_{cat}/K_m depend on the acidic form of an enzyme group with $pK_a \sim 8.5$ with ZGly*p*NP and all other substrates examined (Williams and Whitaker, 1967, and references therein). With substrates for which acylation is thought to be the rate-limiting step, $k_{cat}(=k_2)$ likewise exhibits a pK_a of 8.5 while K_s is essentially independent of pH (Lucas and Williams, 1969). In addition Williams and Whitaker (1967) have shown that k_{cat} $(= k_3)$ is independent of pH from pH 6 to 9.5 with ZGly*p*NP as substrate. In order to see if the rate of acylation determined under conditions of high nucleophile concentration would exhibit pH dependence typical of k_2, the effect of $TrpNH_2$ concentration on the reactions of ZGly*o*NP was examined at a number of pH values. The results shown in Figure 4 indicate that the rate-determining step can be changed at the three pH values

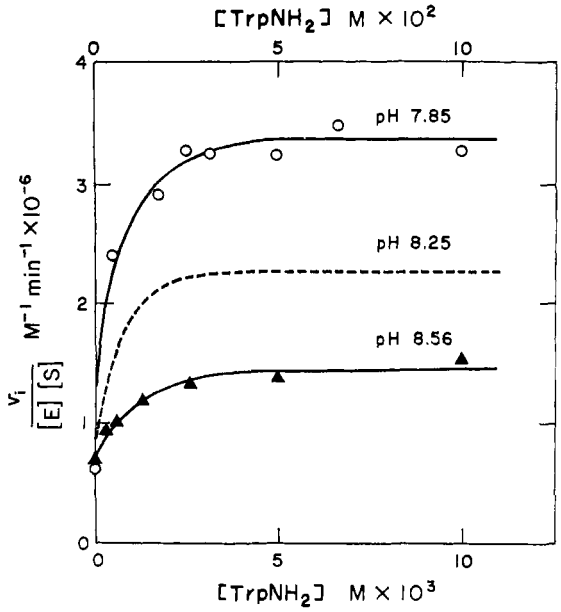


FIGURE 4: The effect of $TrpNH_2$ on the rate of release of *o*-nitrophenol from ZGly*o*NP at various pH values. The upper scale is for points at pH 7.85. The dotted line is from the data in Figure 1. The conditions were: 25°, 9.75% acetonitrile, 0.33 mM ZGly*o*NP, 0.031–0.045 M potassium phosphate buffer (pH 7.85) or 0.045 M sodium borate buffer (pH 8.56), 0.31–0.45 mM EDTA, an ionic strength of 0.41–0.45 (pH 7.85) or 0.45 (pH 8.56).

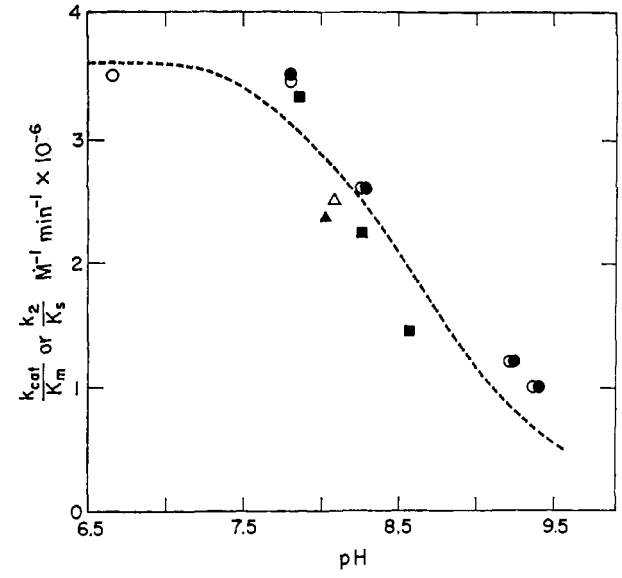


FIGURE 5: The effect of pH on the reactions of ZG1y*o*NP under conditions of rate-determining acylation or deacylation. Filled symbols are data obtained at sufficiently high concentrations of $TrpNH_2$ to ensure that acylation is rate determining. (○,●) Values of $k_2/K_s \cdot (k_{cat}/K_m)$ obtained from first-order plots as described in Figure 3 except that the pH values of the buffers were varied. (△, ▲) Values of k_{cat}/K_m from data in Figure 6 and Table II. (■) Values of $v_{lim}/([E][S])$ from the data in Figure 4. These values are equal to the k_2/K_s because $[ZG1yoNP] << K_s$ and $v_{lim}/([E][S]) = k_2/(K_s + [S])$ (see Table II and text). The curve is theoretical for a pK_a of 8.65 and $k_{cat}/K_m(k_2/K_s) = 3.6 \times 10^6\ M^{-1}\ min^{-1}$.

examined, *i.e.*, the observed initial velocities become independent of $TrpNH_2$ concentration. The values of $v_{lim}/([E][S])$ are markedly pH dependent as expected from the relationship $v_{lim}/([E][S]) = k_2/(K_s + [S])$ which should reflect the pK_a of about 8.5 governing k_2. Values of k_{cat}/K_m were also obtained from first-order data in the presence and absence of $TrpNH_2$, as described above, at a number of pH values. The results obtained by the various methods are shown in Figure 5 where the line is drawn for a theoretical pK_a of 8.65 and a limiting value of k_2/K_s of $3.6 \times 10^6\ M^{-1}\ min^{-1}$.

Dependence of the Rate Constants on Substrate Concentration. Since eq 2 has the form of the Michaelis–Menten equation it is clear that values of k_2, the rate constant for acylation, and K_s, the dissociation constant of the enzyme–substrate complex, can theoretically be obtained from the substrate dependence of the rates of papain-catalyzed hydrolysis of esters at high nucleophile concentrations where $k_{cat}(app) = k_2$ and $K_m(app) = K_s$. The reactions of ZGly*o*NP were therefore examined as a function of ester concentration at 10 mM $TrpNH_2$. This amine concentration is sufficiently high to ensure that the observed rate constant is independent of nucleophile concentration at all substrate concentrations employed (Figure 1) and the conditions required for eq 1 to reduce to eq 2 are met. The results of this experiment are shown in Figure 6. In the presence of 10 mM $TrpNH_2$ the rate of production of nitrophenol is greatly increased at all accessible substrate concentrations and there is no evidence of saturation of observed velocities with substrate at concentrations of up to 0.4 mM ZGly*o*NP. In Table II the calculated lower limits for $k_{cat}(app)$ and $K_m(app)$ at the 90% confidence level are presented.

A similar experiment was carried out with ZGly*p*NP as substrate in the presence of 0.133 M $TrpNH_2$ at pH 8.27. In contrast to the results with ZGly*o*NP, substrate saturation was noted and values of $K_m(app)$ and $k_{cat}(app)$ could be computed

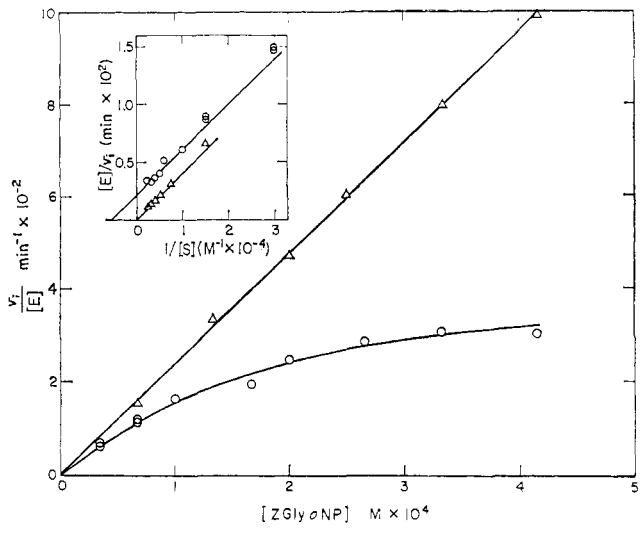


FIGURE 6: The effect of ZG1y*o*NP concentration on the rate of release of *o*-nitrophenol in the presence of $TrpNH_2$. (○) No amine, pH 8.05; (△) 10 mM $TrpNH_2$ (pH 8.10). The conditions were: 25°, 9.75% acetonitrile, 0.045 M sodium pyrophosphate buffer, 0.45 mM EDTA, and ionic strength 0.45. The inset shows a double-reciprocal plot of the same data.

(Table II) under conditions of rate-determining acylation. At the highest substrate concentration employed the rate of deacylation was at least seven times the rate of acylation.[2] Due to the limited solubility of ZGly*p*NP the highest sub-

[2] The total rate of acylation, $k_2[S]/(K_s + [S])$, was calculated using the values given in Table I, and the total rate of deacylation, $k_3' + k_4[N]$, was calculated using the value of k_4 given in the text.

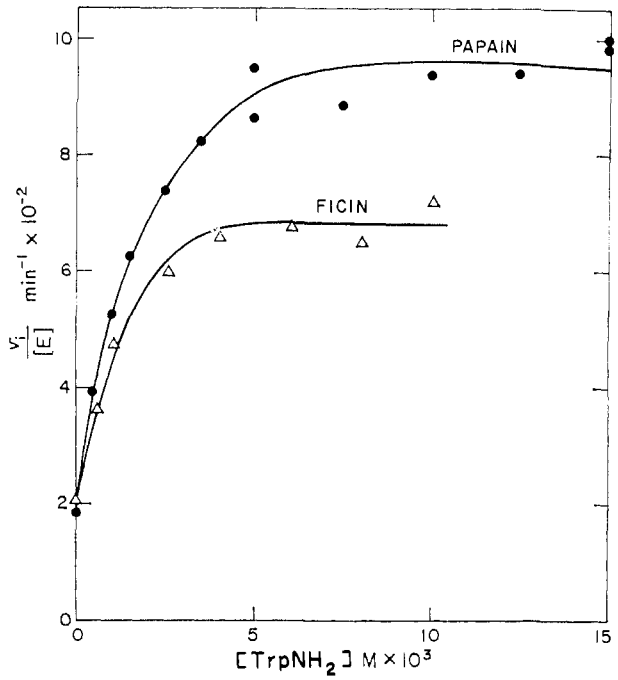


FIGURE 7: The effect of $TrpNH_2$ on the rate of release of *p*-nitrophenol in the papain and ficin catalyzed reactions of *p*NPH. The conditions were as in Figure 1 except the buffer was potassium phosphate and the final pH 7.71 (papain) or 7.80 (ficin). pNPH was 0.1 mM.

strate concentration used (0.2 mM) was only $\sim K_m$(app) so the error in these determinations is large (~25%).

Reactions of Papain and Ficin with pNPH. Hollaway *et al.* (1969) have reported values of k_2 and K_s for the reaction of ficin with pNPH obtained from stopped-flow measurements at [E] ≫ [S].[3] Ficin is very similar to papain in all of its kinetic properties (reviewed by Smith and Kimmel, 1960). It was of interest, then, to study the effect of $TrpNH_2$ on the reactions of ficin and papain with pNPH to learn: (1) if the rate-determining step could be changed and (2) if individual values of k_2 and K_s could be obtained at high nucleophile concentrations under steady-state conditions which would be in agreement with those obtained for ficin by direct measurement. Figure 7 shows the effect of $TrpNH_2$ concentration on the rates of production of *p*-nitrophenol in the papain and ficin-catalyzed reactions of *p*NPH. As was the case with ZGly esters, the observed rate constants increase to a maximum level where they become independent of added nucleophile, suggesting a change in rate-determining step. The reactions of papain and *p*NPH were studied as a function of *p*NPH concentration at three levels of $TrpNH_2$, all of which are on the plateau region of Figure 7. The results shown in Figure 8A indicate that the rates are indeed independent of $TrpNH_2$ concentration at all substrate concentrations. With *p*NPH as substrate, as with ZGly*p*NP, there is evidence of saturation in substrate at high nucleophile concentration where acylation is rate determining. The apparent Michaelis constants are given in Table III. Similar experiments were carried out with ficin at 7.5 mM $TrpNH_2$ and are shown in Figure 8B; the calculated values of K_m(app) and k_{cat}(app) are given in Table III. The values of

[3] An earlier attempt to resolve k_2 and K_s under conditions of [E] ≫ [S] for the papain-catalyzed hydrolysis of ZGly*o*NP at pH 6.8 set a lower limit of $K_s > 10^{-4}$ M (Hubbard and Kirsch, 1968).

TABLE II: Michaelis Parameters Determined under Conditions of Rate-Determining Deacylation and Acylation.

Substrate	ZGly*o*NP[a]	ZGly*p*NP[b]
	Rate-Determining Acylation	
$TrpNH_2$ (mM)	10	133
pH	8.05	8.27
k_{cat}(app) (sec^{-1})	690 ± 775	246 ± 40
K_m(app) (mM)	17.0 ± 19.0	0.199 ± 0.056
k_{cat}(app)/K_m(app) (M^{-1} sec^{-1} × 10^{-4})	3.96 ± 0.03	124 ± 40
90% confidence limits[c]		
k_{cat}(app) (sec^{-1})	≥203	170–325
K_m(app) (mM)	≥6.23	0.118–0.352
	Rate-Determining Deacylation	
pH	8.10	8.25
k_{cat} (sec^{-1})	7.75 ± 0.34	
K_m (mM)	0.184 ± 0.017	
k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-4})	4.20 ± 0.43	59.4

[a] The data for ZGly*o*NP are from Figure 6. The value of k_{cat}(app)/K_m(app) was calculated from a least-squares fit to the data assuming that $v_i/[E] = k_{cat}(app)[S]/K_m(app)$. Values are plus or minus the standard error of the determinations. [b] The data for ZGly*p*NP under conditions of rate-determining acylation were determined at 25° in 0.03 M sodium pyrophosphate buffers containing 0.3 mM EDTA, 9.75% CH_3CN, and ionic strength 0.44; ZGly*p*NP was from 0.02 to 0.2 mM and papain was 20 nM. The value of k_{cat}/K_m was determined at [S] ≪ K_m, from Table I. Values are plus or minus the standard error of the determinations. [c] Confidence limits are based on a one- or two-tailed t test for ZGly*o*NP and ZGly*p*NP, respectively.

k_{cat}(app) and K_m(app) for ficin are reasonably close to those attributed to k_2 and K_s by Hollaway *et al.* (1969) who studied the acylation reaction directly under different conditions of solvent and pH with a different preparation of ficin. Errors in the determinations of K_m(app) and k_{cat}(app) are again large (~25%) because the highest substrate concentration attainable under these conditions (0.4 mM) was only one-half the value of K_m(app). At 0.4 mM *p*NPH and 33.3 mM $TrpNH_2$ the measured initial velocities were shown to be linearly dependent on papain concentration from 19 to 140 nM enzyme.

Determination of k_4. It is possible to obtain a value for k_4, the second-order rate constant for the reaction of nucleophile with the acyl-enzyme, from the dependence of initial velocities on nucleophile concentration and the relationship (from eq 1 and 2):

$$\frac{1}{\frac{[E]}{v_i} - \frac{[E]}{v_{lim}}} = k_3' + k_4[N] \qquad (5)$$

at any constant [S]. It is also possible to obtain a value for k_4 at very high concentrations of substrate and low concentrations of $TrpNH_2$, where $k_2 \gg (k_3' + k_4[N])$ and $(k_2 + k_3' +$

TABLE III: Michaelis–Menten Parameters for the Papain- and Ficin-Catalyzed Reactions of *p*NPH.[a]

Enzyme	[*p*NPH] (M × 10^4)	[Trp-NH_2] (mM)	k_{cat} (sec^{-1})	K_m (M × 10^4)	k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-5})	k_{cat}(app) (sec^{-1})	K_m(app) (M × 10^4)	k_{cat}(app)/K_m(app) (M^{-1} sec^{-1} × 10^5)
Papain	0.05–4.0	0	3.66 ± 0.08	0.317 ± 0.021	1.16 ± 0.08			
Papain	0.40–4.0	10				185 ± 42	9.4 ± 2.8	2.20 ± 0.82
Papain	0.40–4.0	15				173 ± 40	7.6 ± 2.4	2.30 ± 0.90
Papain	0.40–4.0	33.3				180 ± 23	8.9 ± 1.5	2.01 ± 0.43
Ficin	0.10–4.0	0	7.11 ± 0.28	0.680 ± 0.17	1.05 ± 0.12			
Ficin[b]			7.1 ± 0.3	0.530 ± 0.04	1.34 ± 0.17			
Ficin	0.50–4.0	7.5				93 ± 10	7.5 ± 1.1	1.25 ± 0.23
Ficin[b]						44 ± 4	2.8 ± 0.4	1.57 ± 0.40

[a] Values are computed from the data in Figure 8 except where noted. [b] Values from Hollaway *et al.* (1969) at pH 5.9.

$k_4[N])[S] \gg (k_3' + k_4[N])K_s$ and eq 1 reduces to

$$\frac{v_i}{[E]} = k_3' + k_4[N] \quad (6)$$

Initial velocities were obtained at low concentrations of $TrpNH_2$ and high concentrations of ZGly*p*NP and are shown in Figure 9 together with data obtained at lower substrate concentration and plotted according to eq 5. The two methods give essentially the same value of k_4, 4.5 × 10^5 M^{-1} min^{-1}.

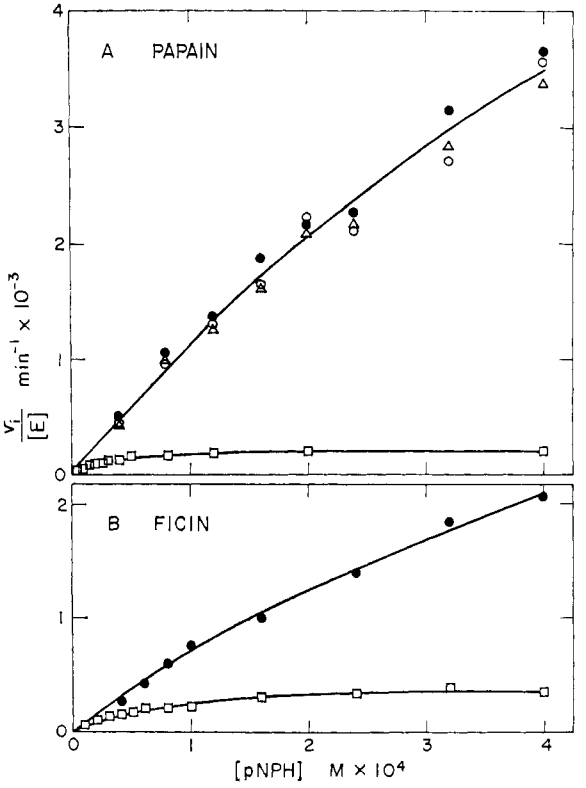


FIGURE 8: The effect of *p*NPH concentration on the rate of release of *p*-nitrophenol in the presence and absence of $TrpNH_2$. (A) Papain + (□) no amine (pH 7.71); (○) 10 mM $TrpNH_2$ (pH 7.71); (●) 15 mM $TrpNH_2$ (pH 7.71); (△) 33.3 mM $TrpNH_2$ (pH 7.85). (B) Ficin + (□) no amine (pH 7.80); (●) 7.5 mM $TrpNH_2$ (pH 7.80). The conditions were: 25°, 9.75% acetonitrile, 0.040 or 0.045 M potassium phosphate buffer, 0.40 or 0.45 mM EDTA, and ionic strength 0.45.

Values of k_4 for the reaction of $TrpNH_2$ with the acyl-papains formed from ZGly*o*NP and ZGly*m*NP were obtained by the same methods and found to be 6.7 × 10^5 and 11 × 10^5 M^{-1} min^{-1}, respectively. It is unclear if the differences in k_4 values obtained with the three esters of ZGly are significant or the result of large errors involved in the determinations.

Discussion

As increasing concentrations of nucleophilic amines are added to reactions of papain and ficin with active ester substrates the initial rates of substrate disappearance increase until a plateau level is reached where the rates are insensitive to further increases in amine concentration. This observation is most easily interpreted as a simple change in the rate-determining step from deacylation to acylation. The limiting velocities obtained in the presence of high concentrations of

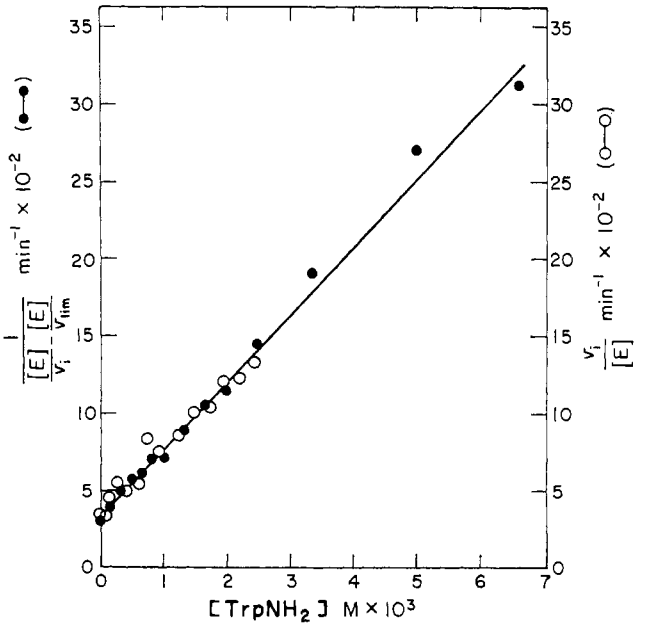


FIGURE 9: Determination of the second-order rate constant (k_4) for the reaction of $TrpNH_2$ with ZGly*p*NP. (●) Data obtained at 0.075 mM ZGly*p*NP and plotted according to eq 5; (○) data obtained at 0.2 mM ZGly*p*NP and plotted according to eq 6. The conditions were otherwise those given in Figure 1.

amines are close to the independently measured rates of acyl-enzyme formation, are the same regardless of the nature of the nucleophile used to change the rate-determining step, and are dependent on an ionizable group with a pK_a of ~8.5 as is k_2, the rate constant for acylation. Michaelis constants determined for the reaction of ficin with *p*NPH under conditions of rate-determining acylation (high [$TrpNH_2$]) are similar to the rate constants obtained directly under presteady-state conditions at [E] ≫ [S] (Hollaway *et al.*, 1969). These results are all consistent with the interpretation that in the presence of high concentrations of amines the rate-determining step for the reactions of papain and ficin with active ester substrates is changed from deacylation to acylation. This demonstration of a change in the rate-determining step demands that an intermediate exist along the reaction pathway for these esters (Jencks, 1964) and lends further support to the acyl-enzyme hypothesis.

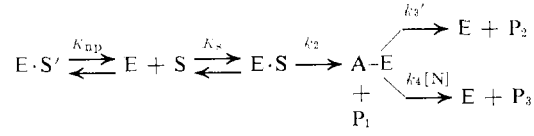
There are, however, a number of observations in the present as well as in prior works which cannot be quantitatively accounted for by the simple two-step mechanism. It is important to note that these discrepancies with the kinetics predicted by the minimal mechanism (Scheme II) are rather small, although they do appear to be beyond the limit of experimental error. Those which arise from the present work are as follows. (1) The ratios of $k_2/K_s(k_{cat}/K_m)$ are increased by $TrpNH_2$ (Table I). The ratio k_2/K_s for ZGly*p*NP increases by a factor of approximately two when 0.1 M $TrpNH_2$ is added. The minimal mechanism (Scheme II, eq 4) predicts that this quantity will not be affected by nucleophiles. (2) Values of k_4 for $TrpNH_2$, determined according to eq 5 and 6, differ by a factor of nearly three for the reactions of the isomeric nitrophenyl esters of ZGly. These rate constants should be identical since they represent the reaction of $TrpNH_2$ with the common acyl-enzyme. (3) From eq 1 it can be shown that at any concentration of nucleophile, k_{cat}(app) must be less than the slowest step in the reaction, but the computed values of k_{cat}(app) for the reactions of ZGly*o*NP and *p*NPH at high concentrations of $TrpNH_2$ (plateau regions of Figures 1 and 8) are greater than the total rate of deacylation ($k_3' + k_4$[N]) calculated with the values of k_4 determined with eq 5 and 6. In the case of *p*NPH, however, k_{cat}(app) appears to be independent of nucleophile concentration: for ZGly*o*NP, k_{cat}(app) > 15,000 min^{-1} and ($k_3' + k_4$[N]) = 7200 min^{-1}; for *p*NPH, k_{cat}(app) = 11,000 min^{-1} and ($k_3' + k_4$[N]) = 4000–13,300 min^{-1} for the three concentrations of $TrpNH_2$ used in the experiments.

Evidence for the Binding of Effector Molecules to Papain. Points 1–3 above cannot be explained by the simple two-step mechanism (Scheme II) for the reaction of proteolytic enzymes with ester substrates. These data, in addition to the known selectivity of papain in reactions with added nucleophiles, suggest that small molecules may bind simultaneously with the substrate at the active site of papain. Evidence for nucleophile binding has been summarized by Fink and Bender (1969), who have proposed that nucleophiles can bind to all forms of the enzyme and thereby alter each of the various rate and binding constants. The mechanism of Fink and Bender (1969) brings into consideration at least two additional rate and one additional equilibrium constants. With the flexibility introduced by these added parameters the points 1–3 above can be quantitatively accounted for. However, further consideration of the present and prior data suggests that the complex scheme required to describe the reactions of papain with small ester substrates might not be required with better substrates expected to bind to a larger region of the active-site cleft of papain (Drenth *et al.*, 1968).

Nonproductive Binding Modes of Small Molecules. There is evidence which suggests that certain small substrates are bound to papain primarily in a nonproductive mode. If the acyl moieties of ZGly and hippuryl esters are bound to the enzyme in the same orientations, then much of the binding energy observed for the *p*-nitrophenyl esters of these acids must reside in the *p*-nitrophenyl group. The reasons for this conclusion are as follows. Reported values of K_m for the papain-catalyzed hydrolysis of the ethyl and methyl esters and amides of ZGly and hippuric acid are between 5 and 270 mM (Smith *et al.*, 1958; Kirsch and Igelström, 1966; Sun and Tsou, 1963; Henry and Kirsch, 1967; Lucas and Williams, 1969). Since under all conditions $K_s \geq K_m$, these values of K_m are *lower* limits for K_s. On the other hand, the results of the present investigation indicate that K_s is between 0.2 and 0.9 mM for the *p*-nitrophenyl esters of these acids. A similar situation obtains with ficin, for which K_m for methyl hippurate is 48 mM (Lowe and Williams, 1965a,b) while K_s for *p*NPH is 0.3–0.9 mM (this work and Hollaway *et al.*, 1969). This means that the apparent binding constants of the *p*-nitrophenyl esters of ZGly and hippuric acid are 10–500 times greater than those of the homologous esters of aliphatic alcohols or amides. In contrast to the results obtained with the *p*-nitrophenyl esters, no indication of saturation of papain by ZGly*o*NP was apparent at substrate concentrations up to 0.4 mM and the minimum values calculated for k_2 and K_s (Table II) were greater than those for ZGly*p*NP. One must conclude that papain is acylated by the ortho-substituted ester at least as rapidly as by the para-substituted derivative but it's dissociation constant is at least 12 times greater. The pK_a values of *o*- and *p*-nitrophenol are nearly identical, and in reactions with hydroxide and mercaptide ions the ortho-substituted esters react somewhat more slowly (Kirsch and Igelström, 1966). The relative rates of acylation of papain by ZGly*o*NP and ZGly*p*NP are not, therefore, those expected on the basis of the reactivity of the esters in model studies. Furthermore, Kirsch and Igelström (1966) obtained evidence which strongly suggested that there are large differences in the rates of acylation of papain by various ZGly esters but much smaller differences in the binding constants.

These considerations, taken together with the apparent capacity of papain to bind substrate and other small molecules simultaneously, suggest the possibility that *p*-nitrophenyl esters bind to papain primarily in a nonproductive mode. The acyl-enzyme scheme, which has been expanded to include nonproductive binding, and the resultant kinetic expressions are shown in Scheme III. Here the complex E·S′ is nonproductive

SCHEME III



and will not react to form an acyl-enzyme intermediate; K_{np} is the dissociation constant of the E·S′ complex. In the absence of nucleophile the steady-state constants for Scheme III are given by

$$k_{cat} = \frac{k_2 k_3'}{k_2 + k_3'[1 + (K_s/K_{np})]} \quad (7)$$

$$K_m = \frac{k_3' K_s}{k_2 + k_3'[1 + (K_s/K_{np})]} \quad (8)$$

In the presence of sufficient nucleophile to change the rate-determining step

$$\frac{v_{lim}}{[E]} = \frac{k_2[S]}{K_s + [S][1 + (K_s/K_{np})]} \quad (9)$$

The Michaelis parameters for the acylation process, measured either directly under presteady-state conditions at [E] ≫ [S] or in the presence of high concentrations of nucleophile, will be

$$k_{cat}(app) = k_2(app) = \frac{k_2}{1 + (K_s/K_{np})} \quad (10)$$

and

$$K_m(app) = \frac{K_s}{1 + (K_s/K_{np})} \quad (11)$$

Some of the consequences of nonproductive binding are as follows. (1) Under conditions of rate-determining deacylation ($k_2(app) \gg k_3'$), the measured values of k_{cat} and K_m will be approximately the same as those which would be observed in the absence of nonproductive binding. If $k_2 > k_3' K_s/K_{np}$ then the existence of nonproductive binding modes will have little effect on the Michaelis parameters. What is important is that if K_s/K_{np} is large then k_{cat} may be considerably less than k_3' even though $k_2 \gg k_3'$. (2) Under conditions of rate-determining acylation low estimates of k_2 and K_s will always be obtained if there is significant nonproductive binding. This will be true whether the acylation reaction is measured under presteady-state conditions or in the presence of high concentrations of amines where $k_2(app) < (k_3' + k_4[N])$.

In terms of this model the *p*-nitrophenyl ester of hippuric acid has a relatively higher affinity for the nonproductive site than the ethyl ester, *i.e.*, $K_s/K_{np}(pNP) > K_s/K_{np}(OEt)$. The significant binding of *p*-nitrophenyl esters in a nonproductive mode can account for the surprisingly low values of K_s and k_2 which were obtained in the present work and by Hollaway *et al.* (1969), and may explain in part the differences in the values of k_4 for the reactions of isomeric nitrophenyl esters of ZGly with $TrpNH_2$.

Brocklehurst *et al.* (1968) have previously shown that postulated nonproductive binding modes could satisfactorily resolve the contradictory conclusions of Whitaker and Bender (1965) and Sluyterman (1968) regarding the rate-determining step in papain-catalyzed reactions of α-*N*-benzoyl-L-arginine ethyl ester if this ester could bind to papain in a mode that did not permit acylation of the enzyme but did increase the reactivity of the active sulfhydryl group toward alkylating agents. The subsequent demonstration of an enhancement in the rate of alkylation of this cysteine residue by the inhibitor α-*N*-benzoyl-D-arginine ethyl ester (Whitaker, 1969) strongly supports this interpretation. While enhancement of the reactivity of a functional group on an enzyme by a substrate is most commonly interpreted as a substrate-induced conformational change in the enzyme (Koshland, 1958), the present results suggest that the elimination of a nonproductive binding mode for the reactant by the substrate will produce the same effect without a conformational change.

The possibility for nonproductive binding of the substrates under consideration here arises because they are small enough to fit into the relatively cavernous active site in more than one orientation. It is known that there are a large number of possible interactions between the enzyme residues in the active-site cleft of papain and the amino acids of large polypeptide substrates (Berger and Schechter, 1970; Wolthers *et al.*, 1970). The former authors have also shown that the selectivity of papain is very high for a substrate bearing a nonpolar side chain on the amino side of the residue undergoing reaction. A schematic diagram showing this interaction together with likely productive and nonproductive binding orientations of ZGly*p*NP is shown in Figure 10. These considerations suggest that many of the apparent kinetic anomalies discussed as points 1 and 3 above, which are due to concurrent binding of substrate and activator (Fink and Bender, 1969; Hinkle and Kirsch, 1970), may arise because of the activator competing with a particular substrate for the dominant nonproductive binding locus from which position the specific rate constants may be increased. It would therefore be predicted that the kinetics of papain-catalyzed reactions of larger substrates, which can bind only in a productive mode, would be substantially free of these complexities.

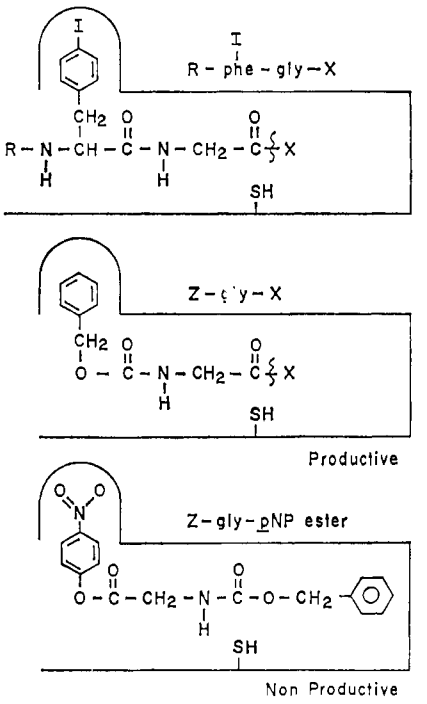


FIGURE 10: Schematic representation of productive and nonproductive binding orientations of ZGly*p*NP compared to the highly productive orientation of R-*p*-IPhe-Gly-X.

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References

Berger, A., and Schechter, I. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 249.

Brocklehurst, K., Crook, E. M., and Wharton, C. W. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 69.

Brubacher, L. J., and Bender, M. L. (1966), *J. Amer. Chem. Soc. 88*, 5871.
Brubacher, L. J., and Bender, M. L. (1967), *Biochem. Biophys. Res. Commun. 27*, 176.
Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M., and Wolthers, B. G. (1968), *Nature (London) 218*, 929.
Englund, P. T., King, T. P., Craig, L. C., and Walti, A. (1968), *Biochemistry 7*, 163.
Fink, A. L., and Bender, M. L. (1969), *Biochemistry 8*, 5109.
Hanson, K. R., Ling, R., and Havir, E. (1967), *Biochem. Biophys. Res. Commun. 29*, 194.
Henry, A. C., and Kirsch, J. F. (1967), *Biochemistry 6*, 3536.
Hinkle, P. M., and Kirsch, J. F. (1970), *Biochemistry 9*, 4633.
Hollaway, M. R., Antonini, E., and Brunori, M. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 299.
Hubbard, C. D., and Kirsch, J. F. (1968), *Biochemistry 7*, 2569.
Jencks, W. P. (1964), *Progr. Phys. Org. Chem. 2*, 63.
Kirsch, J. F., and Igelström, M. (1966), *Biochemistry 5*, 783.
Klein, I. B., and Kirsch, J. F. (1969), *J. Biol. Chem. 244*, 5928.
Koshland, D. E., Jr. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 98.
Lowe, G. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 237.
Lowe, G., and Williams, A. (1965a), *Biochem. J. 96*, 199.
Lowe, G., and Williams, A. (1965b), *Biochem. J. 96*, 189.
Lucas, E. C., and Williams, A. (1969), *Biochemistry 8*, 5125.
Sluyterman, L. A. AE. (1968), *Biochim. Biophys. Acta 151*, 178.
Smith, E. L., Charvé, V. J., and Parker, M. J. (1958), *J. Biol. Chem. 230*, 283.
Smith, E. L., and Kimmel, J. R. (1960), *Enzymes 4*, 133.
Sun, Y.-K., and Tsou, C.-L. (1963), *Sci. Sinica (Peking) 12*, 201.
Whitaker, J. R. (1969), *Biochemistry 8*, 4591.
Whitaker, J. R., and Bender, M. L. (1965), *J. Amer. Chem. Soc. 87*, 2728.
Williams, D. C., and Whitaker, J. R. (1967), *Biochemistry 6*, 3711.
Wolthers, B. G., Drenth, J., Jansonius, J. N., Koekoek, R., and Swen, H. M. (1970), *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., Copenhagen, Munksgaard, p 272.

Regulation of Succinate Dehydrogenase Activity by Reduced Coenzyme Q_{10}*

M. Gutman,† Edna B. Kearney, and Thomas P. Singer‡

ABSTRACT: It is known that, upon combination with substrates or substrate competitors, succinate dehydrogenase is converted from an inactive to active form and that on removal of the activator the enzyme reverts to an inactive form. The activation is characterized by a high energy of activation (36 kcal/mole).

It has been found that during the oxidation of NADH by inner membrane preparations a similar activation of succinate dehydrogenase occurs. The maximal extent of activation reached, the energy of activation of the process, and the kinetic properties of the activated form of the enzyme are the same as when activation is induced by the substrates or substrate analogs. When NADH is exhausted succinate dehydrogenase is rapidly deactivated. The activation–deactivation processes are sufficiently rapid at 37° to be of significance in metabolic regulation. Extraction of endogenous coenzyme Q (CoQ) from the membrane results in loss of activation by NADH but on reconstitution of the particles with respect to CoQ, activation by NADH is restored. These observations and studies with inhibitors indicate that NADH itself is not the activating agent but merely serves to reduce endogenous CoQ and that $CoQH_2$ is the activating agent. It is suggested that $CoQH_2$ is a positive modifier of succinate dehydrogenase. The site at which $CoQH_2$ acts may not be the same as the one involved in electron transport from succinate dehydrogenase to CoQ since thenoyltrifluoracetone abolishes electron flux from the dehydrogenase to CoQ without affecting activation by $CoQH_2$. The role of the activation–deactivation processes in electron flux between the dehydrogenase and the respiratory chain and in the control of the Krebs cycle are discussed.

It has been known since 1955 that succinate dehydrogenase is activated by substrates and substrate analogs which act as competitive inhibitors (Kearney *et al.*, 1955; Kearney, 1957). Thus succinate dehydrogenase was one of the first examples of homotropic regulation discovered, since the succinate serves not only as a substrate but as a positive modifier of the enzyme. The activation has been observed in intact mitochondria, membrane preparations, and the soluble, purified enzyme from a variety of mammalian sources and aerobic yeast cells (Kearney, 1957; Thorn, 1962; Singer *et al.*, 1966) and was found to be reversible upon removal of the activator (Kimura *et al.*, 1967). Two characteristics of the activation process are noteworthy; the high energy of activation (36 kcal/mole), suggestive of a protein modification (Kearney, 1957), and the fact that activation affects only certain activities of the enzyme

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