# Kinetics of the Action of Papain on Fluorescent Peptide Substrates†

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ABSTRACT: Kinetic measurements have been performed on the action of papain on mansyl-Gly-Val-Glu-Leu-Gly and on mansyl-Gly-Gly-Val-Glu-Leu-Gly, both of which are cleaved solely at the Glu–Leu bond under the conditions of our experiments. Stopped-flow experiments have shown that, under conditions of enzyme excess, the enhancement of the fluorescence of the mansyl group upon association of each of the oligopeptide substrates with papain is a biphasic process. A very rapid initial increase in fluorescence is followed by a slower first-order fluorescence enhancement. The observed rate constant for the latter process is greater with the mansyl pentapeptide than with the mansyl hexapeptide. A similar biphasic fluorescence change is seen upon the interaction of the mansyl peptides with mercuripapain, but the second step is much slower than in the case of the active enzyme. The rate of the second step in the association of active papain with the mansyl peptides shows saturation with increasing enzyme concentration, supporting the view that an initial enzyme–substrate complex (ES) is converted in a first-order process to the complex (ES*) that undergoes cleavage to form products. The hydrolysis of the Glu–Leu bond is associated with a first-order decrease in fluorescence, as a consequence of the formation of the mansyl peptide product, which is bound less strongly than the substrate. The rate constant for this process is about 140 times greater with the mansyl hexapeptide than with the mansyl pentapeptide, thus giving further indication of the importance of secondary enzyme–substrate interactions in the efficiency of papain catalysis. For each of the two mansyl peptides, the values of the rate constants and the apparent Michaelis constants associated with the cleavage of the Glu–Leu bond, as determined by stopped-flow measurements under conditions of enzyme excess, were the same, within the precision of the data, as those estimated from experiments under conditions of substrate excess, where the formation of Leu–Gly was determined by means of the fluorescamine reaction. This indicates that, with these substrates, the rate-limiting step in the overall catalytic process is associated with the breakdown of ES*. Estimates are given of the dissociation constant of ES and of the rate constants in the interconversion of ES and ES*.

In a previous publication (Lowbridge and Fruton, 1974) it was reported that papain catalyzes the hydrolysis of Mns-Gly-Val-Glu-Leu-Gly[1] and of Mns-Gly-Gly-Val-Glu-Leu-Gly solely at the Glu–Leu bond under the conditions of our studies. These two substrates bear an amino-terminal fluorescent probe and were designed to examine the interaction of papain with peptide substrates by means of fluorescence spectroscopy. The choice of the oligopeptide structure was guided by the available knowledge of the specificity of papain and the requirement that only a single peptide bond be cleaved by the enzyme. For this reason, the Glu–Leu bond was selected as the site of enzymatic attack, and this dipeptidyl unit was flanked by a valyl residue on the amino side and a glycine on the carboxyl side. To the resulting Val-Glu-Leu-Gly unit, a Mns-Gly or Mns-Gly-Gly group was attached at the amino terminus to examine the effect of secondary interactions, at a distance from the site of catalytic action, on the kinetics of hydrolysis. Under steady-state conditions, the value of $k_{cat}$ for the hexapeptide derivative was found to be much greater than that for the pentapeptide derivative, the apparent $K_m$ values being approximately equal (Lowbridge and Fruton, 1974). This finding suggested that, as in the case of the action of pepsin on oligopeptide substrates (Fruton, 1974), secondary enzyme–substrate interactions may enhance the catalytic efficiency of papain, possibly by inducing conformational changes at the active site. These preliminary studies have now been extended through an examination, by stopped-flow techniques, of the kinetics of the association reaction in the formation of an enzyme–substrate complex in which the mansyl group exhibits enhanced fluorescence, and of the decomposition of the complex, as judged by the decrease in fluorescence. The stopped-flow experiments were performed under conditions of enzyme excess, and the kinetic data were compared with those obtained under comparable conditions under steady-state conditions of substrate excess. Also, since earlier work had shown that mercuripapain binds Mns-Gly-Val-Glu-Leu-Gly and Mns-Gly-Gly-Val-Glu-Leu-Gly strongly, but interacts weakly with mansyl compounds such as Mns-Gly-Gly or Mns-Val-Glu-Leu-Gly (Lowbridge and Fruton, 1974), stopped-flow experiments were performed to determine the rate of association of the first two mansyl peptides with mercuripapain.

## Experimental Procedure

A commercial preparation of crystalline papain (Worthington Biochemical Corp., lots 34D677 and 3AA) was purified by affinity chromatography on a Sepharose-*p*-aminophenylmercuric column in the manner described by Sluyterman and Wijdenes (1970), except that dimethyl sulfoxide, butanol, and KCl were omitted from the 0.05 M acetate buffer (pH 5.0). The eluted enzyme solution (0.05 mM) was concentrated to approximately 15 mg/ml using an Amicon Diaflow apparatus with a UM-10 membrane. The concentrated solution was stored under $N_2$ at 4 °C with a 5 M excess of mercuric chloride. The enzymic activity toward benzoyl-L-arginine ethyl ester was determined titrimetrically using a

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445, 2485 (1966) are: Mns, mansyl, 6-(*N*-methylanilino)-2-naphthalenesulfonyl; ES, enzyme–substrate complex. Unless otherwise noted, the abbreviated designation of amino acid residues denotes the L form.

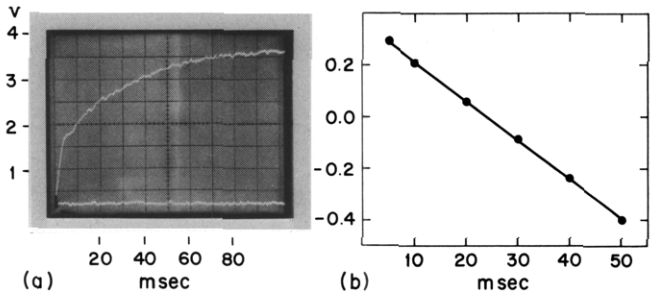


FIGURE 1: Time course of the change in fluorescence during the binding of Mns-Gly-Gly-Val-Glu-Leu-Gly by papain. (a) Oscilloscope trace of the reaction. $[E]_t = 55\ \mu M$, $[S]_0 = 2.5\ \mu M$, pH 6.5 (0.2 M phosphate); 12.5 mM $\beta$-mercaptoethanol; 0.5 mM EDTA; 25 °C. Time constant, 1 ms. The ordinate denotes the change in fluorescence as photomultiplier voltage ($V_t$) in volts (500 mV/division). The bottom trace denotes the end point of the fluorescence change in the overall process. (b) Evaluation of the slower portion of the same time course as a plot of log ($V_e - V_t$) against time, using a value of $V_e = 3.65$ V.

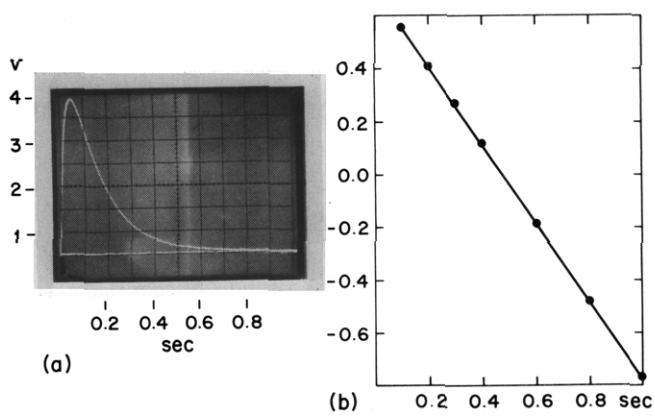


FIGURE 2: Time course of the change in fluorescence during the cleavage of Mns-Gly-Gly-Val-Glu-Leu-Gly by papain. (a) Oscilloscope trace of the reaction. The experimental conditions were the same as in the experiment shown in Figure 1, except for the time scale. Time constant, 10 ms. The ordinate denotes the change in fluorescence as photomultiplier voltage ($V_t$) in volts (500 mV/division). The bottom trace denotes the end point ($V_i$) of the fluorescence change. (b) Evaluation of the decrease in fluorescence as a plot of log ($V_t - V_i$) against time.

Radiometer autotitrator (TTT1a, SBR 2c). The average rate of hydrolysis of 13 mM BzArgEt at pH 6.3, 25 °C (in the presence of 2.3 mM cysteine, 0.5 mM EDTA, and 0.9 KCl) was found to be 28 $\mu M\ min^{-1}\ mg^{-1}$ of enzyme. The enzyme concentration was determined spectrophotometrically at 278 nm using a value of $E^{1\%}_{1cm} = 25.0$ and a molecular weight of 23 700 (Glazer and Smith, 1971). For experiments in which mercuripapain was used without activation, the stock solution was desalted on a Sephadex G-25 column equilibrated with 0.2 M potassium phosphate buffer (pH 6.5). Removal of excess $HgCl_2$ was necessary in order to prevent quenching of the fluorescence of the mansyl compounds.

The kinetic measurements under conditions of $[E]_t \gg [S]_0$ were made with a Durrum Instruments Corp. stopped-flow spectrophotometer (Model D-110) equipped with a Tectronix R-5103N storage oscilloscope, and the photomultiplier tube was mounted at 90° to the tungsten light source. The excitation wavelength was 330 nm, and a Corning 3-72 filter in front of the phototube excluded light below 440 nm. Photomultiplier voltages of approximately 690 V were required with a 3-mm slit width. The stored oscilloscope traces of the time course of change in fluorescence were photographed on Polaroid 107 film. The path length of the reaction cuvette was 20 mm. The temperature of the reagent syringes and reaction cuvette was maintained at 25 °C ±1 °C. After mixing, the initial substrate concentration was 2.5 $\mu M$, and the enzyme concentration ranged from 10 to 100 $\mu M$; $\beta$-mercaptoethanol (10–20 mM) and EDTA (0.45–0.75 mM) were also present, and all reactions were conducted in 0.2 M potassium phosphate buffer (pH 6.5). It was found that complete activation of the mercuripapain used in these experiments required at least a 200-fold molar excess of $\beta$-mercaptoethanol and a 10-fold molar excess of EDTA. For the experiments on the binding of mansyl peptides with mercuripapain, the thiol and EDTA were omitted.

The determination of $k_{cat}$ and $K_m$ under steady-state conditions ($[S]_0 \gg [E]_t$) with fluorescamine as the reagent for the measurement of the rate of formation of Leu-Gly (Lowbridge and Fruton, 1974) was performed at 25 °C, in the presence of 1.25 mM $\beta$-mercaptoethanol and 0.05 mM EDTA in 0.2 M potassium phosphate buffer (pH 6.5). The results accorded with Michaelis–Menten kinetics (least-square linear plots of $v$ (initial velocity) against $v/[S]$). The initial rates of cleavage of the mansyl peptides under steady-state conditions were unaffected by the presence of the higher concentrations of $\beta$-mercaptoethanol (ca. 15 mM) and of EDTA (ca. 0.5 mM) used in the stopped-flow experiments.

Mns-Gly-Val-Glu-Leu-Gly and Mns-Gly-Gly-Val-Glu-Leu-Gly were prepared as described by Lowbridge and Fruton (1974). Separate experiments using thin-layer chromatography showed that, even under conditions of enzyme excess, the only detectable site of cleavage was the Glu–Leu bond, as had been demonstrated previously under conditions of substrate excess.

## Results

Examination of the fluorescence changes in the action of papain on a mansyl peptide substrate under conditions of $[E]_t \gg [S]_0$ showed an initial very rapid increase in fluorescence within the dead time of the apparatus (3–4 ms), followed by a slower first-order increase in fluorescence. A representative oscilloscope trace, together with a semilog plot of the reaction course of the slower process, is shown in Figure 1. There then ensues an even slower first-order decrease in fluorescence (Figure 2). The simplest model to explain these changes is:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-1}^*}{\overset{k_1^*}{\rightleftharpoons}} ES^* \xrightarrow{k_2} E + \text{products} \qquad (1)$$

This model assumes that in the forward reaction there is a very rapid association of the enzyme with the peptide substrate to produce a complex (ES) that is converted in a first-order process to the fluorescent species (ES*) which then undergoes conversion to enzyme and products. The two-step association process in which ES* is formed from E + S should be characterized by a dissociation constant $K_0 = [E][S]/([ES] + [ES^*]) = K_s K_s^*/(1 + K_s^*)$, where $K_s = k_{-1}/k_1$ and $K_s^* = k_{-1}^*/k_1^*$. On the assumption that this model is correct, the results presented in what follows provide estimates for the values of $K_s$, $k_1^*$, and $k_{-1}^*$ in the formation of ES*, and of $K_0$ and $k_2$ in the conversion of ES*. The values of $K_0$ and $k_2$ estimated from stopped-flow experiments under conditions of $[E]_t \gg [S]_0$ for the conversion of ES* to products are compared with the estimates of $K_m$ and $k_{cat}$ obtained under steady-state conditions of $[S]_0 \gg [E]_t$.

*Formation of ES**. Support for the model suggested in eq 1 is provided by experiments in which $[E]_t$ was varied and $[S]_0$ held constant under conditions of $[E]_t \gg [S]_0$. Plots of the observed first-order rate constant ($k^*_{obsd}$) of the slower step in the formation of ES* against $[E]_t$ (Figure 3) show clear

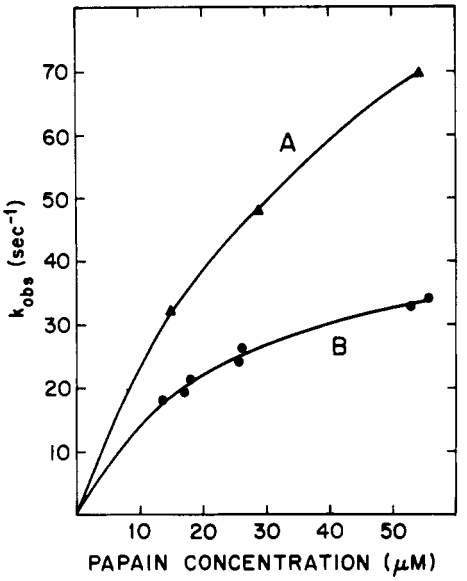


FIGURE 3: Dependence of the observed first-order rate constant of fluorescence increase during association reaction of mansyl peptides with papain on enzyme concentration. Curve A, Mns-Gly-Val-Glu-Leu-Gly; curve B, Mns-Gly-Gly-Val-Glu-Leu-Gly. Substrate concentration, 2.5 μM; pH 6.5 (0.2 M phosphate); 12.5 mM β-mercaptoethanol; 0.5 mM EDTA; 25 °C.

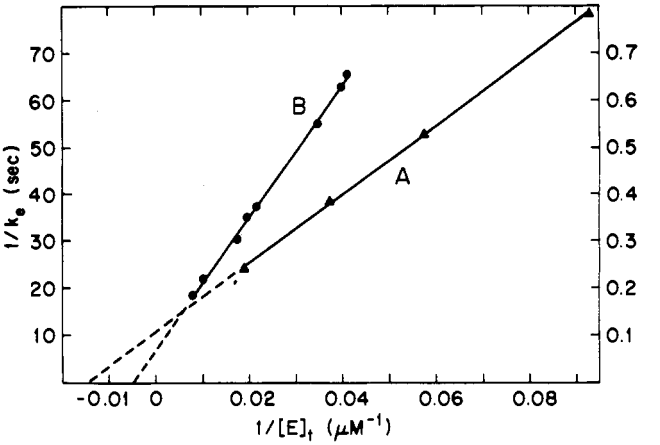


FIGURE 4: Plots of $1/k_e$ against $1/[E]_t$ for the cleavage of mansyl peptides by papain. Curve A, Mns-Gly-Val-Glu-Leu-Gly (left ordinate); curve B, Mns-Gly-Gly-Val-Glu-Leu-Gly (right ordinate). Substrate concentration, 2.5 μM; enzyme concentration, 20–100 μM; pH 6.5 (0.2 M phosphate); 12.5 mM β-mercaptoethanol; 0.05 mM EDTA; 25 °C.

evidence of saturation with increasing enzyme concentration and constant substrate concentration in the case of the mansyl hexapeptide, and a tendency toward saturation with the mansyl pentapeptide.

The two-step association process assumed in eq 1 is analogous to that postulated by Halford et al. (1969) for the binding of the inhibitor 2-hydroxy-5-nitrobenzylphosphonate to alkaline phosphatase from *E. coli*, where a very rapid bimolecular process is followed by a slower first-order reaction; the observed change in the absorbance of the inhibitor upon interaction with the enzyme was attributed solely to the second step. By analogy to their formulation, in the case of the interaction of papain with the two mansyl peptides, the rate of production of ES* from ES under conditions of $[E]_t \gg [S]_0$ may be expected to accord with

$$k^*_{obsd} = k_{-1}^* + \frac{k_1^*[E]_t}{[E]_t + K_s} \quad (2)$$

To calculate the magnitude of $k_1^*$ and $K_s$ from the dependence of $k^*_{obsd}$ on enzyme concentration, a value of $k_{-1}^*$ is required. As will be indicated below, it was possible to make a reasonable assumption regarding the magnitude of $k_{-1}^*$.

*Conversion of ES**. As shown by Kezdy and Bender (1962) for chymotrypsin, under conditions of $[E]_t \gg [S]_0$ the observed rate constant ($k_e$) for release of the first product in the cleavage of a substrate is given by the equation $k_e = k_2[E]_t/([E]_t + K_0)$, where $K_0$ denotes the apparent dissociation constant of the Michaelis complex to E + S. For the process described by eq 1, the above equation becomes

$$k_e = \frac{k_2[1 - (K_0/K_s)][E]_t}{[E]_t + K_0} \quad (3)$$

The data obtained from the stopped-flow measurements of the rate of decrease of fluorescence in the action of papain on the two mansyl peptides were found to accord with eq 3. In Figure 4 are given plots of $1/k_e$ against $1/[E]_t$, and extrapolation gave the estimates of $K_0$ given in Table I; within the precision of the data, they were found to equal the values of $K_m$. It will be noted

TABLE I: Kinetics of Papain Action

| | Mns-Gly-Val-Glu-Leu-Gly | Mns-(Gly)$_2$-Val-Glu-Leu-Gly |
|---|---|---|
| $k_2$ (s$^{-1}$) | 0.10 ± 0.01 | 14 ± 1 |
| $K_0$ (mM) | 0.073 ± 0.010 | 0.20 ± 0.03 |
| $k_{cat}$ (s$^{-1}$) | 0.10 ± 0.02 | 13 ± 1 |
| $K_m$ (mM) | 0.077 ± 0.015 | 0.20 ± 0.06 |

that the intercept on the $1/k_e$ axis gives a value for $k_2$ that is modified by the quantity $[1 - (K_0/K_s)]$, which becomes equal to unity when $K_s \gg K_0$. Since the values of $k_2[1 - (K_0/K_s)]$ obtained from Figure 4 were equal, within the precision of the data, to the values of $k_{cat}$ obtained under conditions of substrate excess (Table I), it appears justifiable to conclude that $k_2 = k_{cat}$, and that $K_s \gg K_0$ for both substrates.

*Rate of Binding of Mansyl Peptides to Mercuripapain.* As noted previously (Lowbridge and Fruton, 1974) the fluorescence of the two mansyl peptides used in this study is enhanced by mercuripapain, and a plot of the fluorescence enhancement against enzyme concentration (at constant concentration of peptide) indicated a dissociation constant ($K_D$) for the mercuripapain–peptide complex of about 0.01 mM for both peptides. Examination of the rate of the formation of the complex by stopped-flow kinetics showed it to be a biphasic process, as with active papain, but the second phase was extremely slow, as compared with the association of the thiol enzyme with the mansyl peptides. Experiments performed at pH 6.5 (0.2 M potassium phosphate buffer) and 25 °C, with 2.5 μM peptide and with mercuripapain concentrations ranging from 5 to 50 μM, gave satisfactory linear plots for the observed first-order rate of the slower increase in fluorescence ($1/k_{obsd}$) vs. $1/[E]_t$. The pentapeptide and the hexapeptide gave similar results for the rate of association (0.15–0.20 s$^{-1}$) and for $K_0$ (0.007–0.008 mM). The latter value is in reasonable agreement with the earlier estimate of Lowbridge and Fruton (1974) for the value of $K_D$ in the association of mercuripapain with the two peptides, suggesting that $k_{-1}^*$ is negligible.

## Discussion

The data in Table I indicate that the estimated values of $k_2[1 - (K_0/K_s)]$ and of $k_{cat}$ are the same, within the precision of the measurements, for each of the two peptides tested. This

supports the view that the rate-limiting step in the cleavage of the Glu-Leu bond is associated with the decomposition of the ES* complex. The question whether such peptide bond cleavage leads to the formation of an acyl-enzyme intermediate is open, in view of the results of Mole and Horton (1973). However, the 130-fold difference in the values of $k_{cat}$ for the two peptides, without comparable differences in $K_m$, gives further evidence for the importance of secondary enzyme–substrate interactions in the action of papain on oligopeptide substrates.

According to eq 2, a plot of $1/(k^*_{obsd} - k^*_{-1})$ against $1/[E]_t$ should intersect the abscissa at $-1/K_s$ and the ordinate at $1/k_1^*$. Since independently determined values of $k_{-1}^*$ were not available, assumptions were made regarding possible values of this parameter, and rough estimates of $K_s$ and $k_1^*$ were made. As noted above, the apparent equivalence of $k_2[1 - (K_0/K_s)]$ and $k_{cat}$ for both peptide substrates indicates that $K_s \gg K_0$. Moreover, since $K_0/K_s = K_s^*/(1 + K_s^*)$, a calculation of the value of $K_s^*$ from the assumed values of $k_{-1}^*$ and $k_1^*$ should provide an indication of the validity of the assumption. Such trials indicated that reasonable values of $k_{-1}^*$ and $K_s$ for both peptides are about 15 $s^{-1}$ and 14 mM, respectively. Plots of $1/(k_{obsd}^* - k_{-1}^*)$ against $1/[E]_t$ gave estimated values for $k_1^*$ of about $4 \times 10^3$ $s^{-1}$ for the mansyl pentapeptide and about $2 \times 10^3$ $s^{-1}$ for the mansyl hexapeptide, corresponding to values of $K_s^*$ of about $4 \times 10^{-3}$ and $8 \times 10^{-3}$, respectively. Substitution of these values in the equation $K_m = K_s/[1 + (k_1^*/(k_{-1}^* + k_2))]$ gave values of about 0.06 mM for the mansyl pentapeptide and about 0.20 mM for the mansyl hexapeptide, in fair agreement with the values determined under conditions of substrate excess (Table I). Admittedly, these estimates of the individual rate constants and of $K_s$ in the association process are tentative, so long as an independently determined value of $k_{-1}^*$ is not available. One means whereby such a determination might be made is by the use of the substrate analogues Mns-$(Gly)_n$-Val-Glu-D-Leu-Gly (n = 1, 2) which may be expected to be resistant to papain action and to be bound to the active site in a manner similar to that of the all-L peptide substrates. Stopped-flow fluorescence experiments in which the complex of papain with such a substrate analogue is mixed with a strong competitive inhibitor of the enzyme, such as acetyl-L-phenylalanylaminoacetaldehyde (Westerik and Wolfenden, 1972), may be expected to give an estimate of $k_{-1}^*$ by measurement of the rate of displacement of the fluorescent ligand from the active site. Such experiments are in progress.

The finding of a biphasic change in fluorescence intensity upon the interaction of the two mansyl peptide substrates with active papain suggests that a very rapid second-order process in which at least some structural elements of the substrate have interacted with the enzyme is followed by a slower first-order process in which conformational changes occur leading to the formation of the enzyme–substrate complex that undergoes decomposition. It is noteworthy that this first-order process ($k_1^*$) is more rapid for the mansyl pentapeptide than for the mansyl hexapeptide, and the hypothesis may be offered that the association reaction involves a very rapid nucleation step in which the specific portion of the substrate (possibly the Val-Glu-Leu unit) binds at the active site, and that this step is followed by a slower conformational change in the substrate during the course of which the mansyl group is drawn into the active site. The general implications of such stepwise association processes between proteins and oligomeric ligands have been discussed by Burgen et al. (1975). In view of earlier data such as those of Holloway and Hardman (1973) on the occurrence of rate-limiting conformational changes in the action of papain on specific substrates, the possibility must also be considered that the two mansyl peptides may also induce such changes at the active site. In this connection, it may be relevant that Drenth et al. (1975) have reported significant changes in the position of active-site amino acid side chains (notably that of His-159) upon interaction of papain with benzyloxycarbonyl-Phe-Ala chloromethyl ketone. It should also be noted that the estimated values of $k_1^*$ in the present studies are in the range ($10^2$–$10^4$ $s^{-1}$) determined for conformational changes in several proteins (Hammes, 1968).

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