Biochemistry

 Volume 17, Number 21 October 17, 1978

Kinetics of Substrate and Product Interactions with Arsanilazotyrosine-248 Carboxypeptidase A†

L. W. Harrison and Bert L. Vallee*

ABSTRACT: The chromophoric intramolecular azoTyr-248·Zn complex detects discrete kinetic steps in the interaction of azocarboxypeptidase with products or substrates that are hydrolyzed slowly. Temperature-jump experiments at 510 nm indicate that the rapid binding of such ligands is followed by a slower change in the conformation of the enzyme–ligand complex:

$$E + L \underset{}{\overset{K_1}{\rightleftharpoons}} EL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (EL)'$$

Analysis of the data generates K_1, the equilibrium constant that defines the initial binding, and the rate constants k_2 and k_{-2} for the forward and reverse steps of this conversion, respectively. For each ligand, the kinetically determined dissociation constant is virtually identical to that obtained at equilibrium from circular dichroic titrations. Although there are small variations in k_2 and k_{-2} for each substrate, all the rate processes are much faster than the rate-determining step for the hydrolysis of these substrates. The proposed model of the mechanism of peptide hydrolysis by carboxypeptidase incorporates the results of these temperature jump experiments.

The changes in local structure that occur synchronously with catalysis are integral features of the catalytic mechanisms of enzymes. Thus, we have employed site-specific inorganic and organic enzyme modifications to introduce spectrokinetic probes that can signal electronic, magnetic, and structural changes that accompany catalysis (Vallee et al., 1971). We have demonstrated previously (Johansen and Vallee, 1971, 1973, 1975; Harrison et al., 1975a) that the intensely chromophoric intramolecular coordination complex between arsanilazotyrosine-248[1] and the zinc atom of azocarboxypeptidase has properties that are extremely useful for mechanistic studies. Thus, the absorption and circular dichroic spectra of the metal complex differ characteristically from those of its constituents; moreover, the probe characteristics of the active enzyme respond dynamically to environmental factors.

The extremely rapid rate of formation and dissociation of the intramolecular complex, measured by temperature-jump and stopped-flow pH-jump methods (Harrison et al., 1975a), is orders of magnitude faster than the rate-determining step in catalysis. Stopped-flow measurements have shown that inhibitors as well as peptide and ester substrates, which are turned over rapidly, disrupt the azoTyr-248·Zn complex before hydrolysis occurs. As a consequence, the spectral properties of the probe signal change in the conformation of azoTyr-248, substrate binding, or other processes relevant to the mechanism of action of this enzyme. Thus, the azo-Tyr-248·Zn complex functions as a spectrokinetic probe of catalytic events.

Detailed temperature-jump experiments now delineate the interaction of substrates or products with azocarboxypeptidase. The present kinetic data show that the rapid binding of substrates or products is followed by a slower change in the conformation of the enzyme–ligand complex. This conformational process is more rapid than the catalytic, rate-determining step and is apparently distinct from yet additional interconversions of enzyme forms observed previously in stopped-flow, pH-jump experiments (Harrison et al., 1975b) which are much slower. The data indicate that a number of different conformational states may play an important role in the mechanism of carboxypeptidase.

Materials and Methods

Carboxypeptidase A_{Anson} (Worthington Biochemical Corp.) was modified with diazotized arsanilic acid according to published procedures (Johansen et al., 1976). Gly-L-Phe, Gly-L-Tyr, Gly-L-Ile, L-Phe, L-Tyr, and L-Ile were purchased from Sigma Chemical Co. L-Benzylsuccinate, obtained by separation of DL-benzylsuccinate, was a gift from Dr. L. Cueni. All other chemicals were reagent grade. Precautions to prevent

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts 02115. *Received May 17, 1978.* This work was supported by Grant-in-Aid GM-15003 from the National Institutes of Health of the Department of Health, Education, and Welfare.

[1] In order to simplify presentation, azocarboxypeptidase refers to zinc monoarsanilazotyrosine-248 carboxypeptidase. AzoTyr-248 and azotyrosine refer to the azophenol of monoarsanilazotyrosine-248 and the azophenolate to its ionized species.

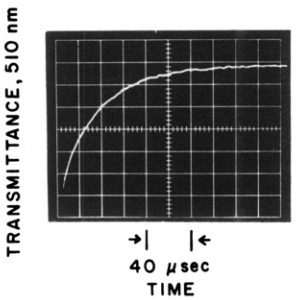

FIGURE 1: Temperature jump of azoTyr-248 carboxypeptidase A and concomitant relaxation effect: enzyme, 20 μM, pH 8.0, 0.1 M Tris-HCl, 1 M NaCl. Initial temperature 21 °C, pulsed by 4 °C to result in a final temperature of 25 °C.

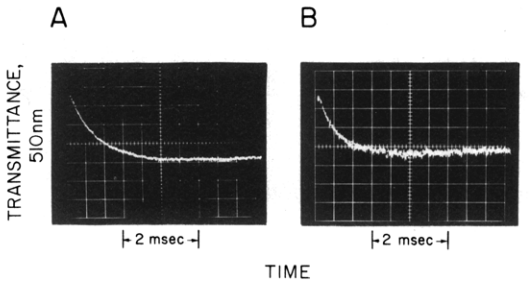

FIGURE 2: Temperature jump of azoTyr-248 carboxypeptidase A in the presence of ligands. (A) 4 mM Gly-L-Phe; (B) 2 mM L-Phe: enzyme 50 μM, 0.1 M Tris-HCl, 1 M NaCl. Initial, final, and temperature pulse as in Figure 1.

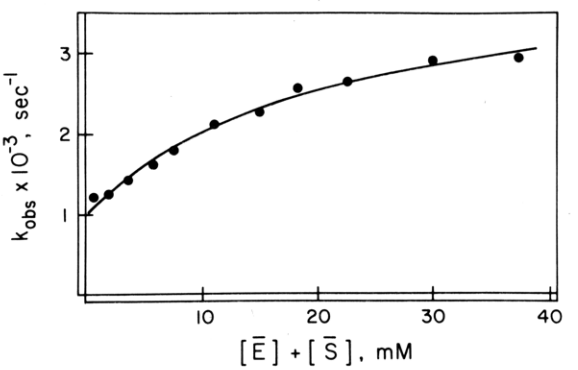

FIGURE 3: Dependence of k_{obsd} on the sum of the concentration of free azoTyr-248 carboxypeptidase A, [Ē], and free Gly-L-Phe, [S̄]: enzyme 50 μM, 0.1 M Tris-HCl, 1 M NaCl. Initial, final and temperature pulse as in Figure 1.

contamination by adventitious metal ions (Thiers, 1957) were taken throughout. Stock solutions of azoenzyme, 5×10^{-4} M, were prepared in 1 mM Hepes,[2] 1 M NaCl (pH 7) and after centrifugation diluted into appropriate degassed solutions prior to temperature-jump experiments. Ligand-binding constants were determined by circular dichroic titration (Johansen et al., 1976).

The essential components and operation of the temperature-jump apparatus have been described (French et al., 1974). In the present version, the heating rate constant is 150 000 s^{-1}. Typically, the signal-to-noise ratio was 10 000:1. A Hewlett-Packard 9810A calculator was used to generate the theoretical curves of Figures 2–4 from best values of parameters in the rate equations, applying nonlinear regression analysis to the mean values of the rate constants.

Results

Stopped-flow experiments have demonstrated that mixing substrates or products with azocarboxypeptidase disrupts the azoTyr-248·Zn complex to form azotyrosine within the 3-ms mixing time, i.e., long before significant hydrolysis can occur (Harrison et al., 1975a). Further details of the interactions of azocarboxypeptidase with a number of slowly hydrolyzed substrates and products have been elucidated using the inherently more rapid equilibrium temperature-jump perturbation method. All experiments were performed at pH 8.0 where the amplitudes of the observed relaxation processes are maximal.

To evaluate the utility of the probe for these studies, the response of the probe itself to temperature change was determined. An azocarboxypeptidase solution, buffered at pH 8.0, was perturbed by temperature-jump employing an instrument with a heating time of 7 μs. On heating, the transmittance at 510 nm increases (Figure 1), indicating the disruption of the red azoTyr·Zn complex to yield yellow azotyrosine. The process is extremely rapid with a rate constant of 29 000 s^{-1}, distinctly different from that for the heating process, 150 000 s^{-1}. It characterizes the only relaxation process of the enzyme alone in this time range. However, in the presence of substrates or products, both this and a second relaxation process are observed.

Temperature-jump experiments have been carried out to examine three slowly hydrolyzed substrates, Gly-L-Phe, Gly-L-Tyr, and Gly-L-Ile, and the corresponding products, L-Phe, L-Tyr, and L-Ile, which are competitive inhibitors. In each instance, measurements were made over as wide a ligand concentration range as feasible and enzyme concentration varied from 10 to 50 μM. When enzyme solutions, each containing a particular ligand, are perturbed by temperature-jump, a characteristic exponential relaxation process in the millisecond time range is observed at 510 nm. For example, in the temperature-jump experiment of azocarboxypeptidase in the presence of the substrate Gly-L-Phe (Figure 2A), the color changes from yellow to red, indicating dissociation of the enzyme–substrate complex and subsequent formation of the azoTyr·Zn chromophore at the higher temperature. The rate constant for this process, 1400 s^{-1}, is about 20 times smaller than that of the response to temperature of the probe itself (29 000 s^{-1}), and is about 100 000 times greater than the value of k_{cat} for this substrate, 0.01 s^{-1}. Thus, the probe clearly measures a process involved in the binding of this substrate. Figure 2B shows a representative oscilloscope trace in the presence of the corresponding product L-Phe. For each substrate or product of this study the transmittance at 510 nm decreases, indicating release of ligand. Concentration-dependent rate constants for these relaxation processes, characteristic of each ligand, range from about 280 to 2000 s^{-1}. The concentration dependences of these rates reveal details of the mechanism of binding for each ligand.

Substrates. The relaxation rate constant of the process, k_{obsd},[3] in the presence of Gly-L-Phe depends markedly on substrate concentration. It ranges from 1200 to 2960 s^{-1} as the concentration of this ligand varies from 0.7 to 38 mM. The dependence of k_{obsd} on the sum of the equilibrium concentrations of free enzyme, [Ē], and the substrate, [S̄], at pH 8, is shown in Figure 3. With increasing concentration, the rate constant increases nonlinearly and appears to approach a limiting value at the highest measurable concentrations. The

[2] Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[3] The relaxation rate constant, k_{obsd}, is equal to the reciprocal of the relaxation time, τ.

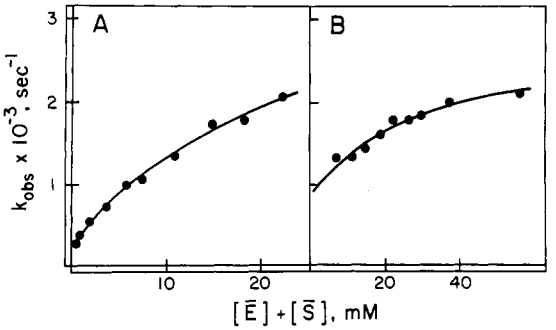

FIGURE 4: Dependence of k_{obsd} on the sum of the concentration of free azoTyr-248 carboxypeptidase A, $[\bar{E}]$, and free substrate $[\bar{S}]$. (A) Gly-L-Tyr; (B) Gly-L-Ile: enzyme 50 μM, 0.1 M Tris-HCl, 1 M NaCl. Initial, final and temperature pulse as in Figure 1.

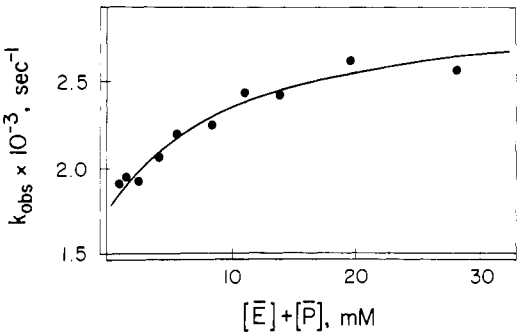

FIGURE 5: Dependence of k_{obsd} on the sum of the concentration of free azoTyr-248 carboxypeptidase A, $[\bar{E}]$, and free L-Phe, $[\bar{P}]$: enzyme 50 μM, 0.1 M Tris-HCl, 1 M NaCl. Initial, final, and temperature pulse as in Figure 1.

TABLE I: Parameters for Ligand Binding to Azocarboxypeptidase.[a]

ligand	k_2 (s^{-1})	k_{-2} (s^{-1})	K_1 (mM)	K_D (mM[b]) kinetic	K_D (mM[b]) equilibr[c]
Gly-L-Phe	3250	1000	21	5.0	5.4
Gly-L-Tyr	4030	271	28	1.8	2.3
Gly-L-Ile	1970	895	32	10.0	11.9
L-Phe	1156	1746	9.9	5.9	4.5

[a] Final conditions: enzyme 50 μM (pH 8.0), 50 mM Tris-HCl, 0.5 M NaCl, 25 °C. [b] $K_D = K_1(1 + k_2/k_{-2})^{-1}$. [c] Determined by titration of the circular dichroic extrema at 510 nm due to the azoTyr-248·Zn complex (Johansen et al., 1976).

TABLE II: Temperature-Jump Relaxation Processes Observed in Azocarboxypeptidase Solutions Containing Inhibitors.[a]

inhibitors	[L] (mM)	k_{obsd} (s^{-1})
competitive		
L-benzyl succinate	0.027	270
L-phenyl lactate	4.0	2260
noncompetitive		
indole 3-acetate	4.0	83
mixed		
phenyl acetate	10.0	820

[a] Final conditions: enzyme 50 μM (pH 8.0), 50 mM Tris-HCl, 0.5 M NaCl, 25 °C.

simplest mechanism consistent with these data is represented in eq 1.

$$E + L \underset{}{\overset{K_1}{\rightleftharpoons}} EL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (EL)' \qquad (1)$$

Here the enzyme and substrate, designated as ligand, L, rapidly associate to form an enzyme·ligand complex, EL. The formation of EL is coupled to a slower, concentration-independent isomerization with another conformation of the enzyme·ligand complex, (EL)′. If the bimolecular association process is assumed to be fast relative to the rate of interconversion of EL and (EL)′, then the observed relaxation rate constant is characterized by eq 2.

$$k_{obsd} = k_{-2} + \frac{k_2}{1 + \dfrac{K_1}{[\bar{E}] + [\bar{L}]}} \qquad (2)$$

The calculated parameters for Gly-L-Phe, Gly-L-Tyr, and Gly-L-Leu are listed in Table I. For Gly-L-Phe, the calculated dissociation constant for the overall reaction [$K_D = K_1(1 + k_2/k_{-2})^{-1}$] is 5.0 mM, in good agreement with 5.4 mM, the value derived from equilibrium measurements. The solid curves in Figures 3 and 4 are calculated from the parameters of eq 2 that result from a nonlinear least-squares fit of the experimental data for all these substrates.

The excellent fit of experimental data points to the calculated curve indicates that the proposed mechanism pertains to all these substrates. Thus, for Gly-L-Tyr (Figure 4A) or Gly-L-Ile (Figure 4B) the nonlinear concentration dependence of k_{obsd} is analogous to that of Gly-L-Phe. The values calculated for the overall dissociation constants agree well with those determined from circular dichroic spectral titrations at equilibrium. Thus, the azoTyr·Zn complex monitors the same elementary step in the binding of each of these substrates, likely a common feature of the mechanism of peptide hydrolysis. There is a similar step for the binding of products.

Products. The products of the hydrolysis of the above substrates, L-Phe, L-Tyr, and L-Ile, are competitive inhibitors of the peptidase activities of carboxypeptidase. The relaxation rate constant associated with the binding of L-Phe (Figure 5) depends on concentration in a manner similar to that of dipeptide substrates (Figures 3 and 4). A fit of the data to eq 2 results in the numerical values of K_1, k_2, and k_{-2} given in Table I. The concentration dependence of the relaxation process due to L-Tyr could not be determined because of the relative insolubility of this ligand. No concentration dependence was observed between 2 and 4 mM, where the rate constant is 820 ± 10 s^{-1}. The relaxation process due to L-Ile is observed only at concentrations above 7 mM, where k_{obsd} is 2000 ± 20 s^{-1}, virtually independent of the L-Ile concentration up to 30 mM. This concentration independence likely reflects an isomerization of the enzyme·L-Ile complex similar to that of L-Phe. In terms of eq 2, this concentration independence implies that for this product k_{-2} is significantly greater than k_2.

Other Inhibitors. Temperature-jump experiments also were performed with azocarboxypeptidase in the presence of L-benzyl succinate, a tightly bound competitive inhibitor; L-phenyl lactate, a competitive inhibitor of ester hydrolysis; indole 3-acetate, a noncompetitive inhibitor of peptide hydrolysis; and phenyl acetate, a mixed inhibitor of the enzyme. In each case, subsequent to the rapid response to temperature of the azoTyr-248·Zn complex itself, a single relaxation process in the millisecond time range was observed at 510 nm. The rate constants for these processes range from about 20 to 2000 s^{-1}, all much slower than the response of the probe to temperature (~10^5 s^{-1}). Representative relaxation rate constants are listed in Table II. Preliminary concentration studies show that, ex-

cept for L-phenyl lactate, the k_{obsd} increases as the concentration of ligand increases. The process due to L-phenyl lactate does not vary with concentration. These experiments indicate that conformational forms of the enzyme·ligand species may also exist in the presence of these ligands. Experiments over a wide range of concentrations are in progress.

Discussion

The kinetic processes that pertain when ligands interact with enzymes have been the subject of many rapid kinetic studies (Hammes, 1974; Hammes and Schimmel, 1970) and, in general, two features have been found in common. The initial binding step is usually very rapid and is often limited by the rate at which ligand and enzyme can diffuse together. This is usually followed by an isomerization of the enzyme·ligand complex, postulated frequently as critical to the catalytic mechanism. The temperature-jump data presented here and previously (French et al., 1974) indicate that different conformational states may also play an important role in the catalytic mechanism of carboxypeptidase.

We have previously detailed the pH dependence of the absorption spectrum of azocarboxypeptidase (Johansen and Vallee, 1971, 1973, 1975). The formation and dissociation of the red intramolecular azoTyr-248·Zn complex are characterized by pK_{app} values of 7.7 and 9.5. The spectrum of the yellow azoTyr-248 begins to predominate below pH 7.7, while that of the orange azophenolate ion appears above pH 9.5. The rates of interconversion of these three species have been determined and have established a basis for studies of mechanism. The conversion of the azoTyr-248·Zn complex to either the azophenol or the azophenolate species was examined in stopped-flow experiments (Harrison et al., 1975a) in which the pH was changed rapidly by mixing weakly buffered enzyme with concentrated buffer solutions at appropriate pH values. The rate of dissociation of the azoTyr-248·Zn complex to either form is more rapid than the 3 ms required for mixing and, hence, faster than the catalytic rate for hydrolysis of substrates. On mixing substrates or products with the enzyme, pH 8.5 (Harrison et al., 1975a), the azoTyr-248·Zn complex is disrupted to form azotyrosine within the 3-ms mixing time, i.e., long before significant hydrolysis can occur. Thus, the azoTyr-248·Zn complex is a sensitive spectrokinetic probe of catalytic events and permits delineation of the interaction of substrates and inhibitors with the enzyme. Now, temperature-jump results further delineate the course of interaction of substrates or products with azocarboxypeptidase.

When Gly-L-Phe, Gly-L-Tyr, or Gly-L-Ile interact with the enzyme, the rapid binding process is followed by a slower interconversion of at least two conformational forms of the enzyme·ligand complex. In terms of this mechanism (eq 1 and 2), the data for each substrate generate rate constants, k_2 and k_{-2}, for the forward and reverse steps of this interconversion, respectively, and, K_1, the equilibrium constant that defines the initial rapid binding step (Figures 3 and 4, Table I). The data obtained with L-Phe, L-Tyr, and L-Ile are consistent with the interconversion of two conformational forms of the enzyme·ligand complex. In particular, the data obtained in the presence of L-Phe (Figure 5) show a concentration dependence that is very similar to that of substrates. The L-Ile rate constant is independent of concentration, and low solubility precludes a detailed concentration study of the L-Tyr process. It is likely that the same conformational equilibria pertain, since the amplitudes and time ranges of these processes are similar to those of other ligands.

While it is not clear that the conformational change for products is identical to that of substrates, each of these inhibitors forms a 1:1 complex with the enzyme. Further, it has been shown (Alter and Vallee, 1978) that the substrate Gly-L-Tyr and the inhibitor L-Phe compete with each other for the same binding site on the enzyme. Thus, it is likely that all the ligands considered here bind at the same site on the enzyme, and it is reasonable to conclude that the kinetic details of this binding are similar in all cases. Comparison of the parameters in Table I emphasizes this similarity. The binding constant for the association step, K_1, ranges from 10 to 32 mM, the forward rate constant, k_2, from 1000 to 4000 s^{-1}, and the reverse rate constant, k_{-2}, from 270 to 1750 s^{-1}. Although these values probably reflect individual differences for each ligand, the variation is quite small. Importantly, all of these rate processes are much faster than the rate-determining step for hydrolysis of these substrates, i.e., 0.01 s^{-1}. Comparison of the overall binding constants for these ligands calculated either from the kinetic data or by direct measurement constitutes a simple test of the mechanism. For each ligand, the kinetically determined dissociation constant (Table I) is virtually identical to that obtained at equilibrium from circular dichroic titrations of the 510-nm band. The rate constants for the conformational processes are also significantly greater than the k_{cat} of more rapidly hydrolyzed peptide substrates (~10–100 s^{-1}). Preliminary stopped-flow, temperature-jump studies with the substrate Cbz-Gly-Gly-Phe (k_{cat} = 55 s^{-1}) have identified a relaxation process similar in amplitude and rate to that for slowly hydrolyzed substrates. Thus, it seems likely that the same mechanism will pertain to all peptide substrates.

While the specific details and role of this conformational change in the catalytic mechanism of carboxypeptidase A are not clear, a tentative assignment of these elementary steps in the overall mechanism of carboxypeptidase can be made. Equation 3, which represents the simplest mechanism of peptide hydrolysis by carboxypeptidase, incorporates the results of these temperature-jump experiments. It assumes that the observed conformations are components of the catalytic reaction and result from productive substrate-binding modes.

$$\mathrm{E} + \mathrm{S} \overset{21\ \mathrm{mM}}{\rightleftharpoons} \mathrm{ES} \underset{1000\ \mathrm{s^{-1}}}{\overset{3250\ \mathrm{s^{-1}}}{\rightleftharpoons}} (\mathrm{ES})'$$

$$(\mathrm{ES})' \xrightarrow{k_{cat} = 0.01\ \mathrm{s^{-1}}} (\mathrm{EP})' \qquad (3)$$

$$(\mathrm{EP})' \underset{1160\ \mathrm{s^{-1}}}{\overset{1750\ \mathrm{s^{-1}}}{\rightleftharpoons}} \mathrm{EP} \overset{10\ \mathrm{mM}}{\rightleftharpoons} \mathrm{E} + \mathrm{P}$$

The rate and equilibrium constants shown refer specifically to the hydrolysis of Gly-Phe. Rapid substrate binding is followed by the conformational process in which ES is converted to (ES)′, followed by substrate hydrolysis and, finally, (EP)′ is converted to EP, followed by product release. Since the rate constants for the conformational processes at the top and bottom are at least five orders of magnitude greater than k_{cat}, i.e., ~1000 s^{-1} compared to 0.01 s^{-1}, these processes are distinct from the rate-determining step.

We have previously demonstrated (Harrison et al., 1975b) the capacity of the intramolecular azoTyr-248·Zn complex to detect multiple conformations of the enzyme in solution. In the absence of ligands, a slow, pH-independent interconversion of two distinct populations of enzyme molecules, E_y and E_y', was identified in stopped-flow pH-jump experiments. This isomerization of the yellow forms of the enzyme is apparently distinct from the processes detected in this study, since its rate constant ($k_{obsd} \sim 5\ s^{-1}$) ranges from 50- to 100-fold less than those listed in Table I. It is apparent from the previous (Harrison et al., 1975b) and present data that azocarboxypeptidase

in solution is characterized by a dynamic equilibrium of multiple conformational states. Detailed examination of the kinetic and equilibrium properties of these states has and will continue to provide new insights into the mechanism of action.

References

Alter, G. M., and Vallee, B. L. (1978), *Biochemistry 17,* 2212.

French, T. C., Yu, N.-T., and Auld, D. S. (1974), *Biochemistry 13,* 2877.

Hammes, G. G. (1974), in *Investigation of Rates and Mechanisms of Reactions* (Hammes, G. G., Ed.) Part 2, 3rd ed, p 147, Wiley, New York, N.Y.

Hammes, G. G., and Schimmel, P. R. (1970), *Enzymes, 3rd Ed., 2,* 67.

Harrison, L. W., Auld, D. S., and Vallee, B. L. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3930.

Harrison, L. W., Auld, D. S., and Vallee, B. L. (1975b), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4356.

Johansen, J. T., and Vallee, B. L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2532.

Johansen, J. T., and Vallee, B. L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2006.

Johansen, J. T., and Vallee, B. L. (1975), *Biochemistry 14,* 649.

Johansen, J. T., Klyosov, A. A., and Vallee, B. L. (1976), *Biochemistry 15,* 296.

Thiers, R. E. (1957), *Methods Biochem. Anal. 5,* 273.

Vallee, B. L., Riordan, J. F., Johansen, J. T., and Livingston, D. M. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 517.

Active Site Directed Irreversible Inhibition of Thermolysin†

David Rasnick and James C. Powers*

ABSTRACT: Thermolysin is irreversibly inhibited at pH 7.2 by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 and 2-(*N*-bromoacetyl-*N*-hydroxyamino)-4-methylpentanonitrile. The inhibition reactions exhibit saturation kinetics with K_I values of 7.5 and 0.80 mM, respectively. Competitive inhibitors of thermolysin (P-Leu-Trp-OK and Z-Phe-OH) hindered the alkylation. The stoichiometry of the reaction was demonstrated to be 0.97 to 1 by use of ^{14}C-labeled $ClCH_2CO$-DL-(N-OH)Leu-OCH_3. No change was observed in the amino acid analysis of the alkylated thermolysin. The inhibitor moiety could be removed with 1 mM NaOH or 1 M NH_2OH, observations which support the existence of an ester linkage between the enzyme and inhibitor. Degradation of thermolysin alkylated with ^{14}C-labeled $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 with CNBr demonstrated that the F_I fragment (residues 121–205) contained the inhibitor moiety. The evidence indicates that the inhibitor binds to the active site of thermolysin with interaction of the hydroxamic acid functional group with the active site zinc atom. Subsequent alkylation of Glu-143 irreversibly inactivates the enzyme. Other alkylating agents lacking the hydroxamic acid such as $BrCH_2CO$-Phe-OCH_3, $BrCH_2CO$-L-MeLeu-OCH_3, and $BrCH_2CO$-L-MeLeu-L-Ala-OCH_3 did not react with enzyme. The inhibitor $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 exhibited considerable specificity and would not inhibit the serine proteases chymotrypsin A_α and subtilisin BPN′, while the neutral metalloproteases A and B from *B. subtilis* were inactivated. Carboxypeptidase A was inactivated very slowly ($t_{1/2} > 3$ days). Haloacetyl-*N*-hydroxypeptides with the appropriate amino acid or peptide structures should be useful for the inhibition of other metalloproteases.

Thermolysin (EC 3.4.24.4) is a neutral metalloendoprotease which requires a zinc atom at the active site for enzymatic activity. The amino acid sequence (Titani et al., 1972b) and the X-ray structure (Matthews et al., 1972a,b; Colman et al., 1972) of the enzyme have been determined. Typical chelating reagents such as ethylenediaminetetraacetic acid and 1,10-phenanthroline inhibit enzymatic activity. A series of potent active site directed reversible inhibitors containing the hydroxamic acid (Nishino & Powers, 1978) and phosphoramidate (Kam & Powers, unpublished results) functional groups have recently been synthesized. The development of these compounds is part of a continuing effort to produce inhibitors for the biologically important metalloendoproteases. A particular example is the enzyme collagenase which has been implicated in the debilitating disease arthritis (Harris & Krane, 1974). Specific irreversible inhibitors of metalloendoproteases could be of potential therapeutic value as well as aiding in the study of the active sites of these enzymes.

Only a limited number of specific active site directed irreversible inhibitors of metalloproteases have been reported. The available inhibitors are directed against either carboxypeptidase A or B. These enzymes are similar to thermolysin both in size and in the catalytic mechanism which requires an essential zinc for enzymatic activity, but differ in cleaving the amide bonds at the carboxy terminal end of peptides. *N*-Bromoacetyl-L-*N*-methylphenylalanine has been shown to modify the essential glutamic acid-270 of bovine carboxypeptidase A_γ^{Leu} (Hass & Neurath, 1971a,b) and bovine carboxypeptidase B (Hass et al., 1972). Carboxypeptidase B is also modified by *N*-bromoacetyl-D-arginine, a reagent that stoichiometrically reacts with the active site glutamic acid residue (Plummer, 1971; Kimmel & Plummer, 1972; Sokolovsky & Zisapel, 1974). The side chains of Tyr-248 and a methionine residue of carboxypeptidase B are modified respectively by bromoacetamidobutylguanidine (Plummer and Kimmel, 1969) and *N*-bromoacetyl-*p*-aminobenzyl-L-succinic acid (Zisapel & Sokolovsky, 1974). To our knowledge no such active site di-

† From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332. *Received March 9, 1978.* This research was supported by Grants HL 18679 and RR 07024-11 (Biomedical Research Support Grant Program) from the National Institutes of Health.

0006-2960/78/0417-4363$01.00/0