BBA 66314

STUDIES ON THE INHIBITION OF NEUTRAL PROTEASES BY 1,10-PHENANTHROLINE

J. FEDER, L. R. GARRETT AND D. KOCHAVI
Monsanto Company, New Enterprise Division, St. Louis, Mo. 63166 (U.S.A.)

(Received December 22nd, 1970)

SUMMARY

1. The inhibition of the thermolysin and *Bacillus subtilis* neutral protease catalyzed hydrolysis of 3-(2-furylacryloyl)glycyl-L-leucine amide (Fa–Gly–Leu–NH_2) by 1,10-phenanthroline has been studied.

2. The mechanism of inhibition appears to involve the removal of zinc from the enzyme to yield inactive apoenzyme.

3. Kinetic analysis of the inhibition curves yielded estimates of the enzyme–metal dissociation constants.

4. Chelator-equilibrated gel filtration columns have proved useful for rapid preparation of apoenzyme.

INTRODUCTION

The bacterial neutral proteases are a group of metallo endopeptidases with a specificity for peptide bonds adjacent to amino acids with hydrophobic side chains. The enzymes isolated from *Bacillus subtilis*[1] and the thermostable thermolysin from *Bacillus thermoproteolyticus*[2–4] as well as the enzyme from *Aeromona proteolytica*[5] have been shown to be zinc enzymes containing one gramatom of zinc per 36 000–40 000 gram molecular weight which is essential for activity. Recently it has been found in our laboratory that the *Bacillus megaterium* neutral protease is also a zinc protease containing one gramatom of zinc per 40 000 grams molecular weight of enzyme[6]. Although the inhibition of these enzymes by a number of chelators has been reported, little has been known as to the mechanism of this inhibition. Studies with 1,10-phenanthroline and the *B. subtilis* neutral protease and thermolysin have indicated that the inactivation is accompanied by a rapid removal of the zinc from the enzyme to yield inactive apoenzyme. These studies are reported here.

EXPERIMENTAL

Materials

The *B. subtilis* NRRLB 3411 neutral protease was isolated from filtration cultures as described[3].

Thermolysin was purchased from Daiwa Kasei Co., Ltd. (Osaka, Japan) and recrystallized from calcium acetate. Hepes buffer (N-2-hydroxyethyl piperazine-N-2-ethone sulfonic acid) was purchased from Fisher Scientific Company and used as such. Only reagent grade salts and distilled water (conductivity below $5 \cdot 10^{-7}$ Ω^{-1}) were used. Particular care to maintain metal free glassware was exercised including repeated soak and rinse with dithizone/carbon tetrachloride prior to use.

The substrate 3-(2-furylacryloyl)glycyl-L-leucine amide (Fa–Gly–Leu–NH_2) was synthesized as described[4].

Sephadex G-25-300 was purchased from Sigma Chemical company. The beads were equilibrated at room temperature with the appropriate buffer chelator solution.

Methods

The enzyme catalyzed hydrolysis of Fa–Gly–Leu–NH_2 was monitored spectrophotometrically at 345 mμ using a Cary 14 PM recording spectrophotometer with a thermostated cell compartment at 25.0 $\pm$ 0.1° as described[4,7].

All reactions were carried out at substrate concentrations much below the K_m ($S_0 = 9.76 \cdot 10^{-4}$ M) giving pseudo first-order kinetics which yielded rate constants, $k = k_{cat}\,(E_0)/K_m$.

The zinc and cobalt content was determined by atomic absorption using a Beckman atomic absorption accessory attached to a Beckman DU spectrophotometer equipped with a 10-inch recorder.

Cobalt thermolysin was prepared from the apoenzyme and $CoCl_2$. Apoenzyme was prepared by either dialysis against $2 \cdot 10^{-3}$ M 1,10-phenanthroline in 0.01 M Tris buffer (pH 7.2)–0.01 M $CaCl_2$ or by gel filtration over Sephadex G-25 equilibrated with $2 \cdot 10^{-3}$ M 1,10-phenanthroline in 0.01 M Hepes buffer (pH 7.2)–0.01 M $CaCl_2$. The phenanthroline was removed by either a second dialysis against the same buffer without chelator or passage over a second Sephadex G-25 column in the same buffer without chelator.

RESULTS AND DISCUSSION

The effect of 1,10-phenanthroline concentration on the *B. subtilis* neutral protease and thermolysin catalyzed hydrolysis of Fa–Gly–Leu–NH_2 is shown in Table I. The observed first order constants ($k = k_{cat}\,(E_0)/K_m$) are shown as a function of inhibitor concentration. Similar inhibition by phenanthroline has been reported by VALLEE and coworkers[2] for thermolysin and by YASUNOBU and coworkers[1] of the *B. subtilis* neutral protease.

The inhibition was instantaneous within the experimental methods employed, no greater inhibition obtained after preincubation with inhibitor than without preincubation. The enzyme inhibition was shown to be reversible upon dilution of chelator enzyme or by addition of excess metal ions. Enzyme containing $2 \cdot 10^{-3}$ M 1,10-phenanthroline when assayed in substrate containing this concentration of chelator was inhibited to greater than 99% (either thermolysin or *B. subtilis* neutral protease). However, if this enzyme solution was added to chelator-free substrate such that the phenanthroline level dropped to about $6 \cdot 10^{-5}$ M all of the enzyme activity was obtained. Likewise, addition of zinc or cobalt to the inhibited enzyme in substrate containing $2 \cdot 10^{-3}$ M 1,10-phenanthroline resulted in total reactivation.

TABLE I

THE EFFECT OF 1,10-PHENANTHROLINE ON THE *B. subtilis* NEUTRAL PROTEASE AND THERMOLYSIN CATALYZED HYDROLYSIS OF Fa–Gly–Leu–NH_2

1,10-Phenanthroline ($\times 10^4$ *M*)	$k \times 10^3$ (sec^{-1})*	
	*B. subtilis neutral protease***	*Thermolysin*†
0	6.05	10.20
0.1	6.70	8.94
0.5	6.50	10.80
1.0	5.81	9.30
2.0	4.28	6.71
2.5	3.85	—
3.0	3.06	4.14
3.5	2.30	—
4.0	1.96	3.39
4.5	1.38	—
5.0	0.97	2.04
10.0	0.07	0.86

* Tris buffer (pH 7.2) 0.1 M; $(S^0) = 9.76 \cdot 10^{-4}$ M; $k = k_{cat}(E_0)/K_m$.
** $(E_0) = 1.33 \cdot 10^{-6}$ M.
† $(E_0) = 1.12 \cdot 10^{-6}$ M.

A similar reversible inhibition was obtained with the *B. megaterium* neutral protease.

The question posed was whether this rapid inhibition was due to the formation of an inactive complex between zinc-enzyme and inhibitor or whether there was a rapid removal of metal from the enzyme yielding an inactive apoenzyme. VALLEE and coworkers[8,9] have demonstrated the predominance of one or the other mechanism with various metalloenzymes.

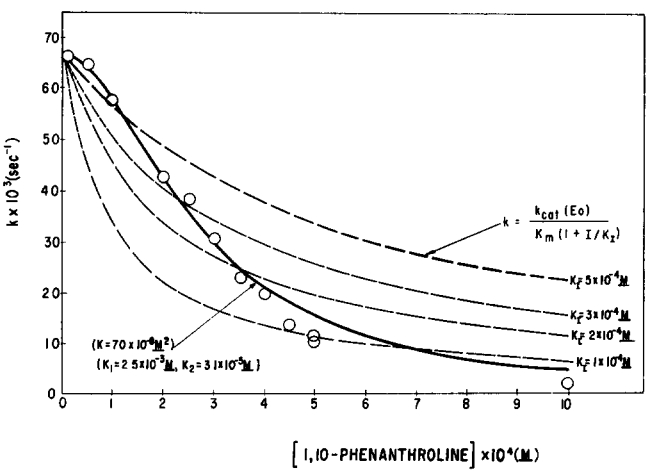


Fig. 1. The effect of 1,10-Phenanthroline on the *B. subtilis* neutral protease catalyzed hydrolysis of Fa–Gly–Leu–NH_2 (details in text). ○, experimental; ———, theoretical line for formation of mixed complex (Eqn. 1 in text); — — —, theoretical curve for single chelator in mixed complex.

If one assumed that a stable enzyme chelator complex was formed, it became apparent that there must be two molecules of the chelator involved in the inhibition. Fig. 1 shows theoretical curves fitted to 1,10-phenanthroline inhibition of the *B. subtilis* neutral protease catalyzed hydrolysis of Fa–Gly–Leu–NH_2 for a single chelator in the complex and for two molecules in the complex. The model used for the latter case is shown below:

$$\begin{array}{ccc} & Em + S \rightleftharpoons Em\cdot S & \xrightarrow{k_{cat}} Em + P \\ + I & \Updownarrow K_1 & \\ & EmI & \\ + I & \Updownarrow K_2 & \\ & EmI_2 & \end{array}$$

Under pseudo first-order rate conditions ($S \ll K_m$) Eqn. 1 was obtained for this mechanism.

$$\frac{dp}{dt} = \frac{k_{cat}\,(Em)\,(S)}{K_m(1 + (I)/K_1 + (I)^2/K_1K_2)} \tag{1}$$

A single phenanthroline molecule in the complex did not fit the data whereas two molecules whether bound sequentially or in one step fit well to yield values of $2.5\cdot10^{-3}$ M and $3.1\cdot10^{-5}$ M for K_1 and K_2 or $7.0\cdot10^{-8}$ M^2 respectively for the chelator enzyme dissociation constants.

In the second mechanism the chelator binds the free metal in solution competing with the enzyme and by mass action the metal is rapidly removed to yield inactive apoenzyme.

The following model would describe this inhibition:

$$\begin{array}{ccccccc} Em + S & \rightleftharpoons & Em\cdot S & \xrightarrow{k_{cat}} & Em + P & & \\ \Updownarrow K_e & & K_1 & & K_2 & & K_3 \\ Em + I & \rightleftharpoons & Im + I & \rightleftharpoons & I_2m + I & \rightleftharpoons & I_3m \end{array}$$

Steady state treatment of this mechanism assuming that all the steps involving metal ion binding by E or I are in equilibrium yield the rate Eqn. 2

$$\frac{dp}{dt} = \frac{k_{cat}\,(E_0)\,(S)}{(S) + K_m\left(1 + \dfrac{K_e}{(M)}\right)} \tag{2}$$

in which (M) is the concentration of free metal ion in solution and K_e is the dissociation constant for the metal enzyme. Assuming that all of the metal is derived from the enzyme $[(E_0) = (M_0)]$ and that the free chelator concentration, (I), is equal to its total concentration and using appropriate equilibrium and conservation relationships the rate equation can be obtained in terms of (E_0) and (I). Under pseudo first-order rate conditions ($S \ll K_m$) Eqn. 3 was obtained.

$$\frac{dp}{dt} = \frac{k_{cat}\,(E_0)\,(S)}{K_m\left[1 + \dfrac{2}{\left(\sqrt{1 + \dfrac{4(E_0)}{K_e A}} - 1\right)}\right]} \tag{3}$$

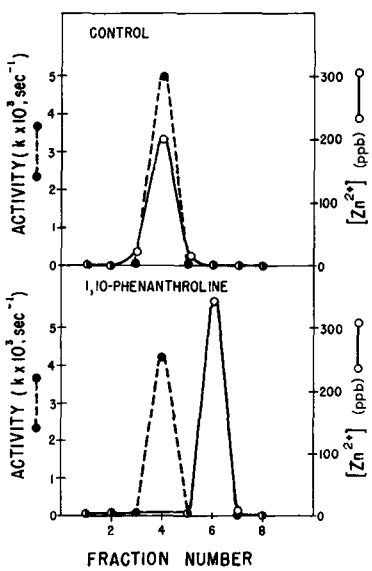
$$A = 1 + I/K_1 + I^2/K_1K_2 + I^3/K_1K_2K_3$$

As demonstrated below theoretical curves for this model also fitted well to the

experimental data. If the latter mechanism was responsible for the inhibition, then one could obtain some estimate of the value of the enzyme metal dissociation constant from the inhibitor concentration rate data. In either case an initial examination of the data indicated that there was a dependence upon at least two molecules of chelator for the inhibition.

An attempt was made to make use of gel filtration techniques to determine whether one or the other mechanism were in operation. Chromatography of heat denatured *B. subtilis* neutral protease or thermolysin over Sephadex G-25 in 0.1 M Hepes buffer (pH 7.0) yielded a protein peak at the void volume with retardation of the zinc upon the column. Likewise, 1,10-phenanthroline was strongly retarded on these columns. Native enzyme, of course, yielded enzyme peaks which exhibited enzymatic activity and zinc superimposed upon the protein peak. Thermolysin and the *B. subtilis* neutral protease were passed over short columns (15–20 cm × 1 cm) of Sephadex G-25 equilibrated with $2 \cdot 10^{-3}$ M 1,10-phenanthroline in 0.1 M Hepes buffer (pH 7.2). If the phenanthroline formed a stable mixed complex, the zinc would be eluted with the enzyme peak in the void volume. However, if the metals were rapidly removed it would be retained on the column. These columns, which ran very rapidly (about 5–10 min), yielded enzyme peaks which had greater than 95% of the zinc removed. Fig. 2 shows the chromatographic elution patterns for the passage of *B. subtilis* neutral protease over Sephadex G-25 with and without $2 \cdot 10^{-3}$ M 1,10-phenanthroline. The phenanthroline column enzyme had to be reactivated by metal in the substrate. Thermolysin yielded similar results.

It was also observed that the metal ion in the neutral protease could be exchanged by addition of high concentrations of a competing metal ion. Cadmium was



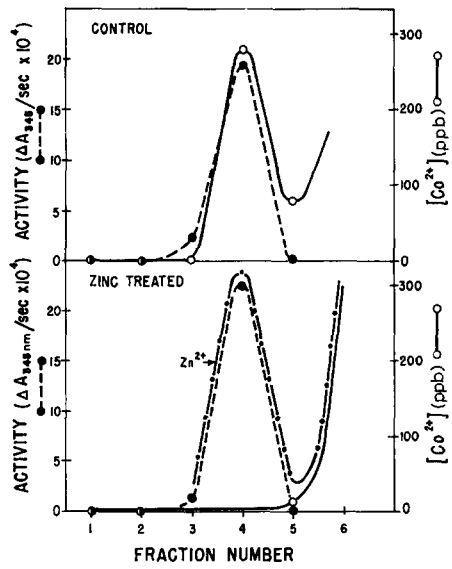
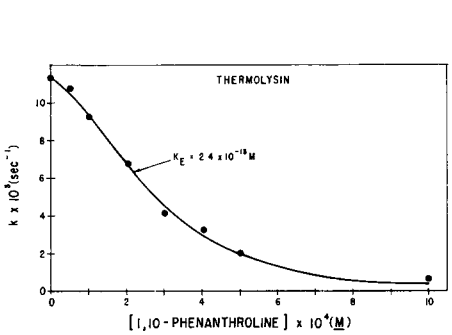


Fig. 2. Effect of 1,10-phenanthroline on the Sephadex G-25 gel filtration of *B. subtilis* neutral protease (details in text). ppb, parts/billion.

Fig. 3. Displacement of cobalt from cobalt-thermolysin by zinc (details in text).

shown to displace the zinc in thermolysin. To a solution of the zinc-thermolysin was added Cd^{2+} to a concentration of $1 \cdot 10^{-3}$ M. This was passed over a Sephadex G-25 column equilibrated with 0.1 M Hepes buffer (pH 7.2) and both zinc content and enzyme activity were determined. Over 50% of the zinc was removed as compared with the control experiment without added cadmium. Fig. 3 shows the exchange of cobalt from cobaltthermolysin by zinc ions. The cobalt enzyme exhibited a cobalt peak coincidental with the activity when passed over Sephadex G-25 in 0.1 M Hepes buffer (pH 7.2). Addition of excess zinc (about $1 \cdot 10^{-4}$ M) followed by gel filtration over this column resulted in the disappearance of the cobalt from the enzyme peak and its replacement by a zinc peak. These experiments which indicate the dissociation of enzyme to apoenzyme and metal would suggest that the 1,10-phenanthroline inhibits the neutral protease by removal of the metal to form inactive apoenzyme (Eqn. 3) rather than by the formation of a mixed complex.



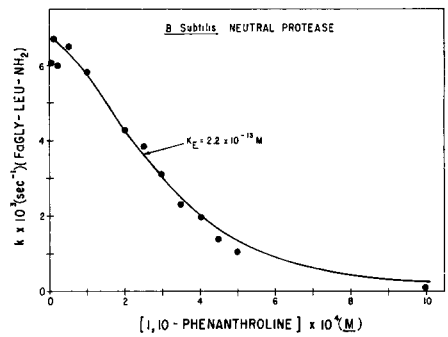


Fig. 4. Inhibition of thermolysin neutral protease by 1,10-phenanthroline. ●, experimental; ———, theoretical curve as described by Eqn. 3 (details in text).

Fig. 5. Inhibition of *B. subtilis* neutral protease by 1,10-phenanthroline. ●, experimental; ———, theoretical curve as described by Eqn. 3 (details in text).

Figs. 4 and 5 show the fit of the theoretical curve described by Eqn. 3 to the experimental results for thermolysin and *B. subtilis* neutral protease. The enzyme concentration was determined to be $1.12 \cdot 10^{-6}$ M for thermolysin and $1.33 \cdot 10^{-6}$ M for the *B. subtilis* enzyme. Values of $3.46 \cdot 10^{-7}$, $2.36 \cdot 10^{-6}$ and $8.2 \cdot 10^{-6}$ for K_1, K_2 and K_3 respectively, were used[10].

The value of K_e was varied to give the best fit using a Fortran IV program to calculate the curves. Under these conditions values of K_e of $2.4 \cdot 10^{-13}$ M and $2.2 \cdot 10^{-13}$ M were obtained for thermolysin and *B. subtilis* neutral protease respectively. A preliminary examination of the inhibition of the 1,10-phenanthroline inhibition curves for the *B. megaterium* neutral protease catalyzed hydrolysis of furylacylglycyl–L-leucine amide yielded K_e value of the same order of magnitude. The inhibition of the cobalt-thermolysin by 1,10-phenanthroline fitted the theoretical curve as defined by Eqn. 3 and yielded a K_e of $3 \cdot 10^{-10}$ M which is about 0.001-fold that for the zinc-enzyme (Fig. 6). Values of $5.24 \cdot 10^{-8}$, $2.0 \cdot 10^{-7}$ and $1.12 \cdot 10^{-6}$ were used for K_1, K_2 and K_3, respectively.

These results do not exclude the preliminary formation of a mixed complex

of phenanthroline–enzyme which rapidly dissociates to yield apoenzyme. A simple model for such a scheme is shown below:

$$\begin{array}{ccccccc} & & K_E & & & & k_{cat} \\ E + & m & \rightleftharpoons & Em + S & \rightleftharpoons & Em\cdot S & \longrightarrow Em + P \\ & \Updownarrow K_1 + I & & \Updownarrow K_L & & & \\ E + & mI & \rightleftharpoons & EmI & & & \\ & \Updownarrow K_2 & & & & & \\ & mI_2 & & & & & \\ & \Updownarrow K_3 & & & & & \\ & mI_3 & & & & & \end{array}$$

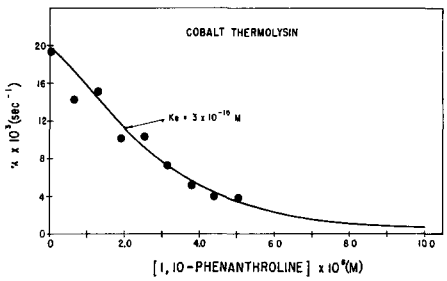


Fig. 6. Effect of 1,10-phenanthroline on the cobalt-thermolysin catalyzed hydrolysis of Fa–Gly–Leu–NH_2. ●, experimental; ———, theoretical curve as described by Eqn. 3.

Under pseudo first-order rate condition with similar assumption as for Eqn. 3, the following rate expression (4) was obtained:

$$\frac{dp}{dt} = \frac{k_{cat}\,(E_0)\,(S)}{K_m\left[1 + \dfrac{2(1 + I/K_L)}{\left(\sqrt{1 + \dfrac{4(E_0)\,(1 + I/K_L}{K_e\,A}} - 1\right)} + I/KL\right]} \qquad (4)$$

$$A = 1 + I/K_1 + I^2/K_1K_2 + I^3/K_1K_2K_3$$

Fig. 7 shows the fit of the theoretical curve described by Eqn. 4 to the experimental results for the effect of 1,10-phenanthroline on the thermolysin catalyzed hydrolysis of Fa–Gly–Leu–NH_2. Values of $2.4 \cdot 10^{-13}$ M and $1 \cdot 10^{-2}$ M for K_e and K respectively were used to calculate this curve. Likewise, values of $2.2 \cdot 10^{-13}$ M and $5 \cdot 10^{-3}$ M were obtained for K_e and K_L respectively for the *B. subtilis* neutral protease. Little

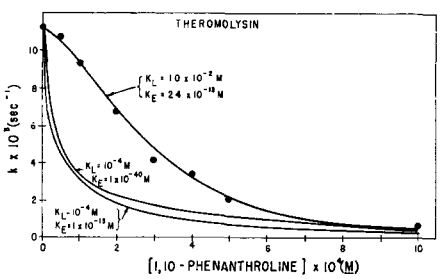


Fig. 7. Inhibition of thermolysin by 1,10-phenanthroline. ●, experimental; ———, theoretical curve as described by Eqn. 4 (details in text).

difference in the estimated values of K_e were thus obtained by introducing the intermediate complex or neglecting it. One cannot differentiate between the mechanism involving the intermediate complex (Eqn. 4) and the one without it (Eqn. 3).

These results with the bacterial neutral proteases are similar to what was reported by VALLEE and coworkers[8] for the inhibition of carboxypeptidase A by 1,10-phenanthroline in distinction to the formation of mixed complexes with horse liver alcohol dehydrogenase[9].

In conclusion the analysis of inhibition curves of chelators with metalloenzymes could be used to give an estimate of the enzyme–metal dissociation constants. The use of gel filtration involving chelator equilibrated columns yielded a simple rapid method for the preparation of apoenzymes. This was particularly useful with the *B. subtilis* neutral protease which was so susceptible to autolytic and other degradation during metal removal by other methods.

REFERENCES

1 J. D. MCCONN, D. TSURU AND K. T. YASUNOBU, *J. Biol. Chem.*, 239 (1964) 3706.
2 S. A. LATT, B. HOLMQUIST AND B. L. VALLEE, *Biochem. Biophys. Res. Commun.*, 37 (1969) 33.
3 L. KEAY, *Biochem. Biophys. Res. Commun.*, 36 (1969) 257.
4 J. FEDER AND J. M. SCHUCK, *Biochemistry*, 9 (1970) 2784.
5 T. B. GRIFFIN AND J. M. PRESCOTT, *J. Biol. Chem.*, 245 (1970) 1348.
6 L. KEAY, J. FEDER, L. R. GARRETT, M. H. MOSELEY AND N. CIRULIS, *Biochim. Biophys. Acta*, in the press.
7 J. FEDER, *Biochem. Biophys. Res. Commun.*, 32 (1968) 326.
8 J. P. FELBERT, T. L. COOMBS AND B. L. VALLEE, *Biochemistry*, 1 (1962) 231.
9 B. L. VALLEE AND T. L. COOMBS, *J. Biol. Chem.*, 234 (1959) 2615.
10 L. S. SILLEN AND A. E. MARTEL, *Stability Constants of Metal-Ion Complexes*, The Chemical Society, Burlington House, London, 1964, p. 665.