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STRUCTURAL AND FUNCTIONAL DETERMINANTS OF *MUCOR MIEHEI* PROTEASE VI

INACTIVATION OF THE ENZYME BY DIAZOACETYL NORLEUCINE METHYL ESTER, PEPSTATIN AND 1,2-EPOXY-3-(*p*-NITROPHENOXY)PROPANE

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Summary

Mucor miehei protease (EC 3.4.23 —), an acid protease of fungal origin, was rapidly inhibited at pH 5.0 and 10°C by a 78-fold molar excess of diazoacetyl norleucine methyl ester (N_2Ac-Nle-OMe) when simultaneously added with a 78-fold molar excess of Cu(II). Preincubation with Cu(II) before the addition of N_2Ac-Nle-OMe reduced the initial rate of activity loss presumably due to a copper-induced structural change as deduced from an examination of CD spectra. Complete inactivation was associated with the incorporation of 1.6 ± 0.12 residues of norleucine and 1.02 ± 0.041 mol of copper. The conformation of the N_2Ac-Nle-OMe-inhibited enzyme appeared to be somewhat altered since the rate of H-^{3}H exchange determined for the slowest exchanging class of hydrogens was reduced by more than 10-fold although the estimated number of hydrogens in this class remained constant.

Mucor miehei protease was also inhibited by pepstatin; complete inactivation required a 6-fold molar excess of inhibitor and was associated with a major conformational change as determined from CD spectra. Loss of activity also occurred in the presence of 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP).

Introduction

Most acid proteases (EC 3.4.23 —), irrespective or origin, are inactivated by diazoacetyl norleucine methyl ester (N_2Ac-Nle-OMe) in the presence of copper; loss of activity being associated with the esterification of a unique aspartic acid residue [1]. Consequently, inactivation by N_2Ac-Nle-OMe and a second residue specific reagent, 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) are now consid-

ered as reactions typical of enzymes which are optimally active at acid pH [2, 3]. However, there are several examples of acid protease such as the enzymes from *Aspergillus niger* Type A and *Scytalidium lignicolum* which are not inhibited by either compound or the *Streptomyces* pepsin inhibitor "pepstatin" [4, 5]. Consequently, at least two types of acid proteases may be distinguished; the pepsin-like, which are sensitive to inhibition by N_2Ac-Nle-OMe, EPNP and pepstatin, and the remainder, resembling the "A" enzymes of *Sc. lignicolum* which are not inhibited by one or more of these three compounds. *Mucor miehei* protease, unlike most other acid proteases, is a glycoprotein resembling the "A" enzymes of *Scytalidium* in carbohydrate content, isoelectric point and molecular weight [6,7]. In addition recent studies of the amino acid sequence about the basic disulfide loop of the *Mucor* enzyme did not reveal any obvious homologies with the aspartate gastric proteases [8]. One aim of the present study was to establish whether this enzyme is, in fact, an aspartate enzyme as defined by the inhibition studies referred to earlier.

A second aim was to investigate the role of Cu(II) in the N_2Ac-Nle-OMe reaction, since the presence of a multi-fold excess of copper might be expected to result in a modification of protein structure in addition to stabilizing the proposed carbene intermediate [7]. For example, a hydrogen-tritium exchange study of conalbumin has shown that copper binding is effective in retarding isotope exchange indicating a "tighter" structure while a calorimetric investigation of the interaction of copper and albumin has demonstrated that the binding of copper results in a general loosening of the native structure [9,10]. A similar diversity of response has been reported with respect to the effect of copper on enzymatic activity. In the presence of a 3000—4000 molar excess of Cu(II) the proteolytic activity of *Scytalidium* acid protease was increased 200% while the proteolytic activities of the acid proteases from *Physarum polycephalum* and *Mucor pusillus* were reduced 80 and 48%, respectively [4,11,12].

Materials and Methods

M. miehei protease was isolated from the commercial product "Rennilase" provided by Novo Industri A/S, Copenhagen, Denmark; the details of the isolation procedure and the characteristics of the purified enzyme have been described [7,13]. Pepstatin was a generous gift from Dr. P. Corvol and Dr. T. Hofmann while EPNP was purchased from Eastman Organic Chemicals.

[2-^{14}C]Glycine, specific activity 650 μCi/mg was obtained from Amersham; L-norleucine, glycine and carbobenzoxyglycine from Sigma Corp.; carbobenzoxychloride, 95%, from Aldrich Chemicals and dicyclohexylcarbodiimide from Fluka A/G. Triethylamine was distilled and stored over sodium.

[2-^{14}C]Glycyl-L-norleucine methyl ester hydrobromide was prepared by removing the benzyloxycarbonyl group of the coupled product from carbobenzoxyglycine and L-norleucine methyl ester by standard methods. Diazotization was carried out as described by Rajagopalan et al. [14] and resulted in a product which was chromatographically pure with properties similar to those recorded earlier.

Tritiated water was purchased from ICN Chemical and Radioisotope Division and had a specific activity of 100 mCi/g.

Analytical methods

1-mg samples of protein were hydrolyzed at 110°C for 24 h under N_2 in a sealed tube and analyzed using a Beckman 121C amino acid analyzer. The amino acid composition including norleucine content was established by a comparison with results determined under similar conditions [7,14,15]. The amount of norleucine incorporated was also established from the specific activity of labelled protein solutions measured using a Beckman LS-133 liquid scintillation counter with sufficient counting time being allowed so that a relative standard error of 1.5% was recorded for the lowest-counting samples. Tritium activity was determined in a similar fashion.

Estimates of the copper content of protein solutions were obtained using a Perkin-Elmer 460 atomic absorption spectrometer equipped with a graphite furnace. Sample volumes of 20 μl containing about 40 μg of protein were dried at 100°C for 60 s, ashed at 920°C for 30 s, and atomized at 2500°C for 7 s. The sensitivity of the instrument for copper under these conditions was such that atomization of an aqueous solution of copper nitrate containing $2.5 \cdot 10^{-3}$ μg of copper/ml resulted in an absorbance of 0.0044. Quantitation was based on six determinations using the method of standard additions in order to minimize protein matrix effects.

Repetitive circular dichroid (CD) spectra were determined using a Cary Model 61 recording spectropolarimeter after the addition of a 78-fold molar excess of copper acetate at 4°C; conditions which approximate those used in the reaction of *M. miehei* protease with N_2Ac-Nle-OMe described below. Other conditions were as described previously [16].

Estimates of proteolytic activity were obtained using acid denatured hemoglobin as substrate at 6—8 different protein concentrations with and without preincubation in the presence of a 78-fold molar excess in copper.

The methods used in the hydrogen-tritium exchange study of the N_2Ac-Nle-OMe-inhibited enzyme were essentially as previously described with the exchange-in reaction being carried out at the desired pH for 24 h in the presence of 1 mCi of tritium [13].

Reaction with pepsin inhibitors

There has been little agreement as the exact conditions to be used in investigations of the reaction of N_2Ac-Nle-OMe with various acid proteases. Although the pH of the reaction mixture was generally between pH 5.0 and 5.6, the N_2Ac-Nle-OMe : Cu(II) ratio, the order of reagent addition, the reagent : protein ratio, and the temperature employed in the reaction vary significantly [2, 14,17—19]. In the present investigation, a protein concentration of 1 mg/ml in 0.05 M acetate buffer pH 5.0, was maintained in all experiments as well as Cu(II) : N_2Ac-Nle-OMe : protein ratio of 78 : 78 : 1. Generally N_2Ac-Nle-OMe was added immediately after the copper acetate as a 0.15 M methanolic solution. When a reaction mixture was required, it consisted of equal quantities of a 0.3 M methanolic solution of N_2Ac-Nle-OMe and 0.3 M copper acetate preincubated for various times in 0.05 M acetate buffer, pH 5.0. Temperature was maintained at 10 ± 1°C by means of water-jacketed reaction vessel and a circulating water bath.

The course of the inactivation was followed by removing 10-μl aliquots of

the reaction mixture for activity determinations at 1-min intervals after the N_2Ac-Nle-OMe addition. Since the reaction was rather rapid, an equal volume of 1.0 M citrate buffer, pH 4.0, in some instances, was added which arrested the inactivation so that larger quantities of labelled protein could be prepared for the H-^{3}H exchange studies, estimations of copper content and amino acid analyses. These "quenched" solutions were exhaustively dialyzed at 4°C against seven changes of double-distilled water over a period of 4 days and then lyophilised.

In some experiments, first the enzyme was totally inactivated by incubation at 38°C for 30 min at pH 5.0 with 6 M guanidine; then the temperature was reduced to 10°C and the appropriate amounts of copper acetate and N_2Ac-Nle-OMe added. In other experiments the enzyme was inactivated by the addition of a 6-fold molar excess of pepstatin prior to incubation with N_2Ac-Nle-OMe.

The extent of pepstatin inhibition at 25°C was estimated from the residual activity after 30 min of solutions containing 1.0 mg of enzyme on 0.5 M acetate buffer, pH 5.0, and varying amounts of a 0.5% solution of the inhibitor dissolved in 0.01 M NaOH. The conformation of the enzyme under conditions of partial and total inhibition was also investigated by obtaining CD spectra in the presence of varying amounts of inhibitor. Exactly 30 min after pepstatin addition, scans were made and the spectra compared with that of the native enzyme reported previously [16].

In a separate series of experiments, sufficient pepstatin was added to solutions of the enzyme to produce a 50% loss in activity and the residual activity determined at various time intervals in order to obtain some information with respect to the time dependency of the reaction.

Inactivation with EPNP was accomplished essentially as described by Tang [3]. A 2000-fold molar excess of solid EPNP was added to an enzyme solution at 30°C containing 1.5 mg of protein/ml in 0.1 M acetate buffer, pH 3.8. The solution was stirred vigorously to ensure an even suspension of the reagent and aliquots, corresponding to about 1.5 mg, taken at appropriate times and freed of excess reagent by gel filtration using a different 16 × 0.9 cm column of Sephadex G-15 equilibrated with the reaction buffer for each aliquot. Aliquots of the recovered protein were taken for estimates of proteolytic activity and EPNP incorporation. The resultant enzymatic activities are expressed as a percentage of a control solution without EPNP but otherwise treated in an identical fashion.

The number of incorporated EPNP residues was estimated from

$$\text{Residues EPNP} = (A_{315\text{nm}} \cdot 4.8)\,[A_{280\text{nm}} - (A_{315\text{nm}} \cdot 0.438)]$$

as suggested by Tang [3].

Results

Modification by N_2Ac-Nle-OMe

M. miehei protease was rapidly inactivated at 10°C by a 78-fold molar excess of N_2Ac-Nle-OMe and Cu(II) when both reagents were added simultaneously; the "half-time" for complete inactivation being about 3 min.

Preincubation of solutions of N_2Ac-Nle-OMe and Cu(II) for 20 min did not

result in a significant modification of the course of the reaction but a marked difference was observed when the enzyme solution was preincubated for 20 min with Cu(II) prior to the addition of N_2Ac-Nle-OMe. Under these conditions, the "half-time" was increased approx. 3-fold to about 10 min while the time required for >95% inactivation (approx. 20—25 min) remained unaltered (see Fig. 1).

The complex nature of this reaction is further illustrated by the insert in Fig. 1 in which the data are presented in a semi-logarithmic plot. Although a quantitative evaluation was not attempted, it is obvious that the initial phase of the reaction was quite rapid but slowed considerably after about 3 min so that the final rate of activity loss was less than 30% of the initial. This initial rapid loss of activity followed by a much slower rate of inactivation appears to be a general characteristic of the reaction. For example, although the time taken for 50% inactivation of pepsin at 14°C and pH 5.6 in the presence of a 66-fold molar excess of Cu(II) and 10-fold molar excess of N_2Ac-Nle-OMe was about 6 min [2], under almost identical conditions; the half-times for the inactivation of chymosin and *Mucor* rennin have been reported as about 20 and 45 min, respectively, while the time required for complete loss in activity was about 5 h [20]. This discrepancy might be due to differing salt contents in the protein preparations since it is obvious from Fig. 2 that with respect to *M. miehei* pro-

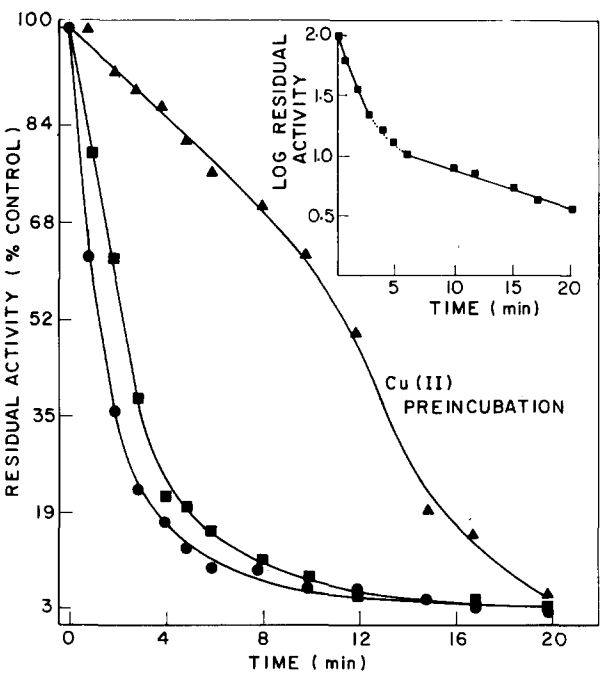
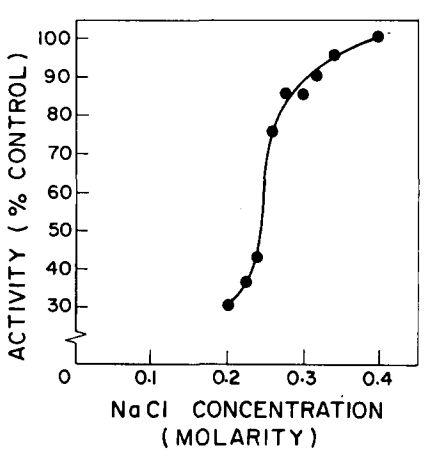


Fig. 1. Representative data indicating the loss of enzymatic activity at pH 5.0 and 10°C in the presence of a 78-fold molar excess of N_2Ac-Nle-OMe (DAN) and cupric acetate (see text). ●———●, N_2Ac-Nle-OMe and Cu(II) acetate were preincubated for 20 min at 10°C prior to addition to the enzyme solution; ■———■, aliquots of N_2Ac-Nle-OMe and Cu(II) acetate were added simulataneously; ▲———▲, the enzyme solution was preincubated for 20 min with Cu(II) acetate prior to the addition of N_2Ac-Nle-OMe. The insert is a semi-logarithmic plot of data obtained from the simultaneous addition of N_2Ac-Nle-OMe and Cu(II) acetate.

tease, at least, the loss of proteolytic activity was very much dependent on NaCl concentration.

The data presented in Fig. 3 demonstrate that norleucine incorporation was directly related to the loss of enzymatic activity throughout most of the reaction. The initial 70% loss in activity, which takes about 6—8 min at 10°C, was associated with the incorporation of about one residue of norleucine as estimated from amino analyses. Complete inhibition resulted in the incorporation of an addition of 0.6 residue for a total of about 1.6 ± 0.12 residues (12 independent experiments) with similar results being obtained based on estimates of ^{14}C incorporation. These values are not unusual and, in fact an incorporation of more than one residue of norleucine would seem to be the rule rather than the exception in such studies [2,4,18]. The relationship between that final 30% loss in activity and norleucine incorporation was difficult to determine since the enzyme which had been inactivated by 6 M guanidine still incorporated about 0.5 residue of label as did that pepstatin-inhibited enzyme. It is also interesting to note that the N_2Ac-Nle-OMe-inhibited protein contained 1.02 ± 0.04 mol of copper per mol of protein while less than 0.01 mol per mol of protein were found in the native enzyme.

The CD spectra in Fig. 4A, taken at 5°C and various times after the addition of a 78-fold molar excess of copper, show an obvious time dependency in ellipticity at 232 and 245 nm and suggested a copper-mediated conformational change. This fact is perhaps a bit clearer in Fig. 4B in which $[\theta]_{232}$ and $[\theta]_{245}$ are plotted as a function of time. The value for $[\theta]_{245}$, decayed to that of the native enzyme within the time taken for the rapid phase of the N_2Ac-Nle-OMe reaction which was about 10 min (see Fig. 1). On the other hand $[\theta]_{232}$ did not return to its initial value until about 20 min after the addition of copper and continued to decrease. The loss of the band centered at 232 nm has been noted



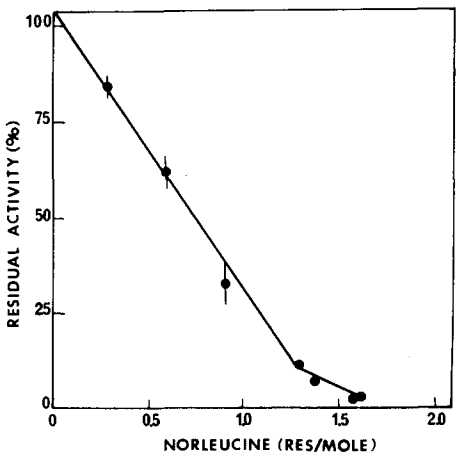


Fig. 2. Inhibition of the N_2Ac-Nle-OMe (DAN) reaction by increasing amounts of NaCl. The experimental conditions are identical to those described under Fig. 1 (■———■) with the exception that the reaction mixture contained varying amounts of NaCl.

Fig. 3. The relationship between loss of activity and norleucine incorporation under standard conditions (■———■), Fig. 1.

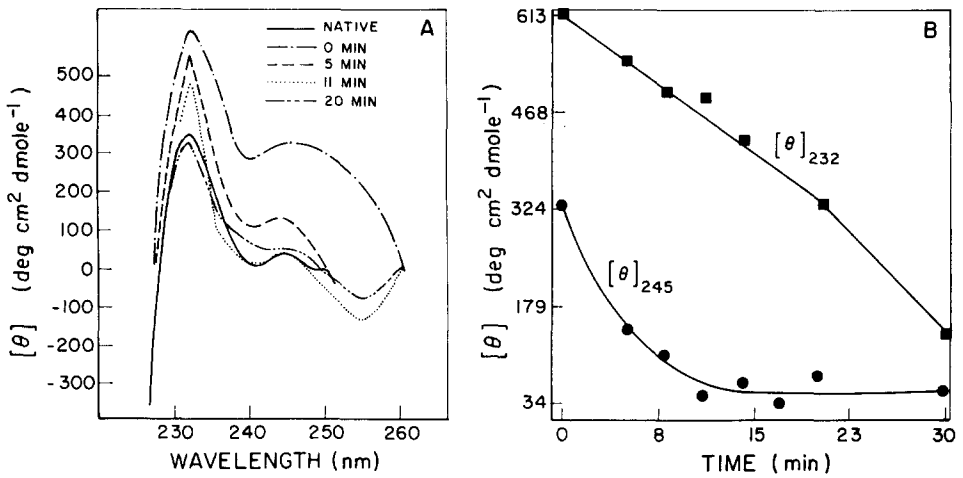


Fig. 4. The effect of copper on the CD spectra of the enzyme at pH 5.0 and 5°C. (A) The time-dependent change in the ellipticity. Repetitive scans were made between 227 and 260 mm following the addition of Cu(II) at the times indicated in the figure. (B) The decay of the ellipticities at 245 and 232 nm as a function of time following the addition of copper. Initial values at 245 and 232 nm were 43 and 332 degree · cm^{-2} · $dmol^{-1}$, respectively.

previously both after exposure to 6 M guanidine and prolonged periodate oxidation and was attributed to loss of asymmetry about one or both disulfide linages [16]. The region of the spectra ascribed to peptide transitions (below 224 nm) was essentially the same as reported previously for the native enzyme indicating that a major change in conformation had not occurred.

When the N_2Ac-Nlc-OMe-inhibited enzyme was subjected to tritium exchange under conditions identical to those employed previously for the native enzyme, the kinetics of the exchange-out reaction were significantly altered as early as 20—35 min after removal of bulk tritium. At this point, the slope of the curve suggests a decreased solvent accessibility to all but the fastest exchanging group of peptide hydrogens. A value of $1.77 \cdot 10^{-4}$ min^{-1} was estimated for the slowest measurable rate as compared with a value of $20.3 \cdot 10^{-4}$ min^{-1} determined previously for the native enzyme [13]. Since the number of hydrogens exchanging with this rate constant, as estimated from the data in Fig. 5 was unchanged, N_2Ac-Nle-OMe inhibition did not cause a major conformational change.

The effect of bound copper on the net charge of the enzyme was also ruled out as a possible explanation for the observed rate retardation on the following grounds. Based on previously published titration data [13], the presence of 1 mol of copper per mol of protein should have the effect of reducing the net charge at pH 5.0 from −10 to −8 so that the resultant exchange-out kinetics would be expected to resemble those of the native enzyme at pH 4.83. Assuming that the effect of copper was purely electrostatic, the magnitude of the resultant decrease in rate constant would be about 35%, which is not sufficient to account for the difference in the "slow" rates (Fig. 5). Consequently, the results are interpreted as indicating a general tightening of the structure of the enzyme which might be related to copper binding (see Discussion).

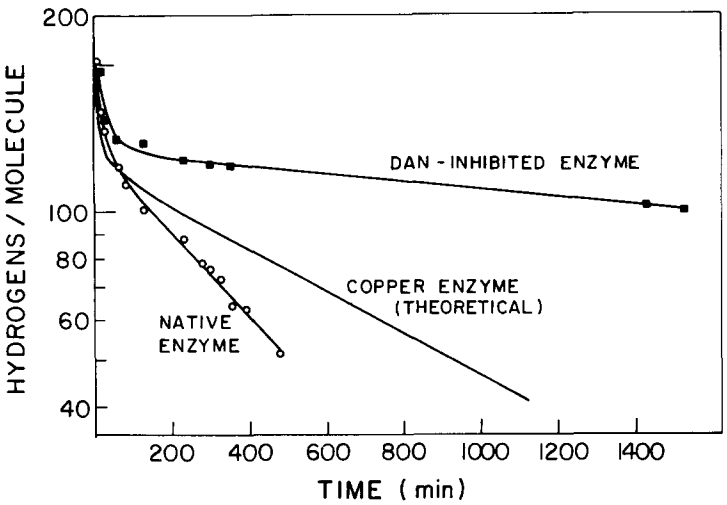


Fig. 5. Exchange-out curves for the native and N_2Ac-Nle-OMe (DAN)-inhibited enzymes at 0°C and pH 5.0. Included in the figure is the theoretical curve generated for the copper-containing enzyme assuming only an electrostatic effect and the presence of 1 mol of copper per mole of protein.

Pepstatin inhibition

M. miehei protease was progressively inhibited by increasing amounts of pepstatin when incubated at pH 5.0 for 30 min at 25°C (Fig. 6A). The hypothesis of a one to one correspondence between activity loss and amount of inhibitor present was not supported by these results since extrapolation of the initial rate of inactivation indicated that about 1.5 mol of pepstatin would be required for complete inhibition; in actual fact, about 6 mol were required. Evidently at about 30% residual activity, the progress of the reaction changed dramatically so that 15 times more pepstatin was required for the elimination of the residual 30% as for the initial 30% of the activity. A similar result was reported by Saheki and Holzer [21] from their investigation of the inhibition of yeast protease A by pepstatin and Miller et al. [22] from a similar study of kidney renin. In contrast Kunimoto et al. [23] have reported that the binding of 1 mol of pepstatin resulted in complete inhibition of pepsin, and suggested that pepstatin might be useful as an active site titrant. Based on the present results, and those obtained by others, the use of pepstatin in this way would seem to have a limited potential. Also, the case of *M. miehei* protease, it is obvious from Fig. 6B that partially inhibited solutions of enzyme regained significant amounts of activity after prolonged incubation which makes a more quantitative interpretation of the data difficult.

Furthermore, a complete explanation for pepstatin-induced activity loss must also consider the effect of conformational changes on the kinetics of inhibition. Fig. 7 shows the CD spectra of solutions containing 40 μg of pepstatin (Fig. 7A), equimolar quantities of pepstatin and enzyme (Fig. 7B) and a 6-fold molar excess of pepstatin over enzyme (Fig. 7C). The spectra in Figs. 7B and 7C were corrected for the pepstatin contribution and, consequently, provide some information with respect to the conformation of the enzyme under these

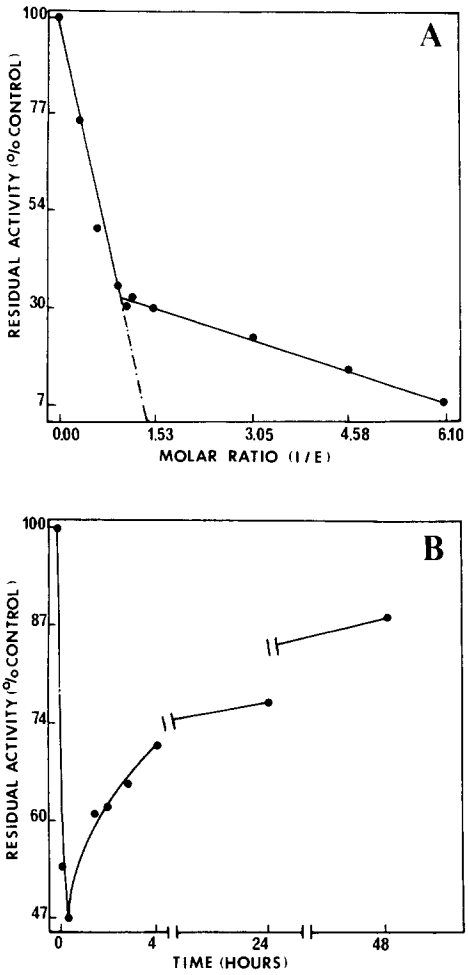


Fig. 6. (A) The progressive inhibition of *M. miehei* protease after 20 min incubation at pH 5.0 and 25°C in the presence of varying amounts of pepstatin. (B) Regeneration of proteolytic activity after prolonged incubation of a solution of enzyme containing 0.61 mol of inhibitor per mol of enzyme.

conditions since pepstatin can be considered as having no organized structure (Fig. 7A).

As noted earlier, 30 min after the addition of an equimolar amount of pepstatin, there was a 70% loss in proteolytic activity which is not associated with a major conformational change although the CD spectrum under these conditions suggested that there was a general "loosening" of tertiary structure (Fig. 7B). Specifically, the shape of the curve was identical to that of the native enzyme but the magnitude of the ellipticity has been reduced. In contrast, the addition of a quantity of pepstatin sufficient to totally inhibit the enzyme did produce a major conformational change (Fig. 7C). The minimum in the CD band normally ascribed to peptide transitions has been shifted from 212 to 217 nm with the concomitant appearance of broad and poorly resolved shoulders at 208 and 225 nm and a large increase in ellipticity at 232 nm. The shift in the

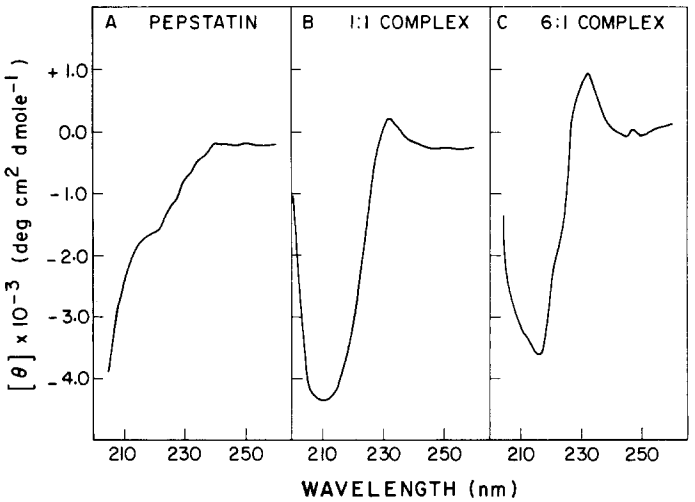


Fig. 7. The CD spectra at 25°C of a solution of pepstatin and solutions containing varying amounts of pepstatin and enzyme. A, pepstatin, 40 μg/ml in acetate buffer pH 5.0; B, a solution containing equimolar quantities of pepstatin and enzyme; C, a solution containing a 6-fold molar excess of pepstatin (see Fig. 6A and text for additional details).

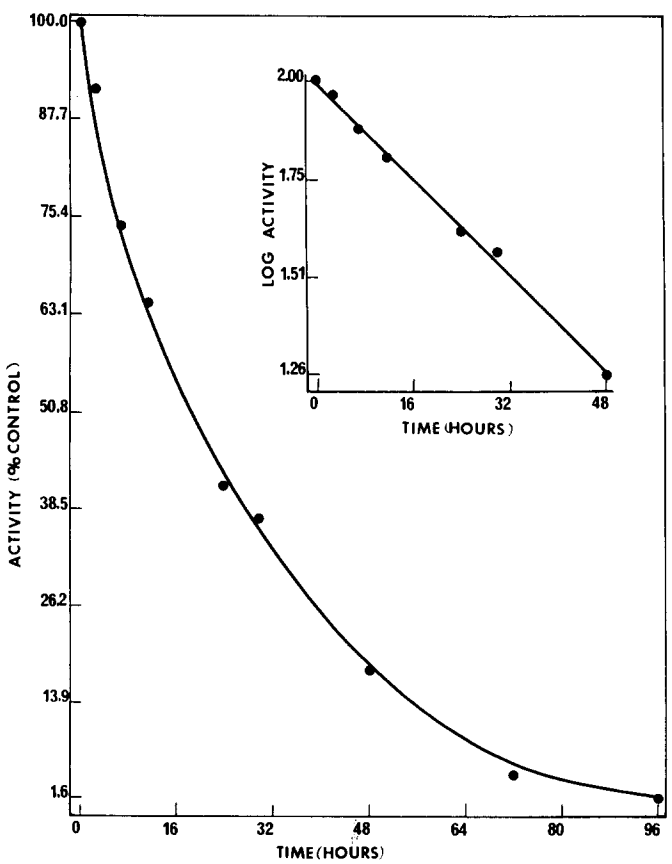


Fig. 8. Inactivation of *M. miehei* protease at 30°C in 0.1 M acetate buffer, pH 3.8, containing a 2000-fold molar excess of EPNP.

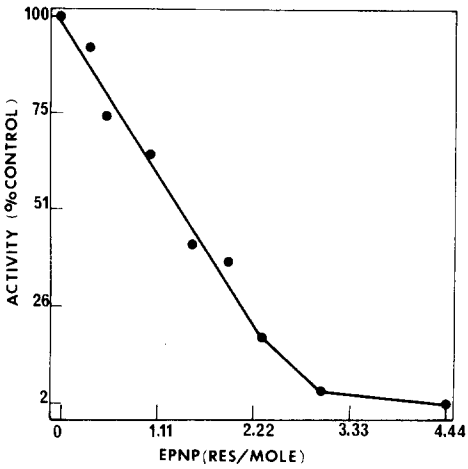


Fig. 9. The relationship of activity loss to residues of bound EPNP. The experimental conditions were identical to those described below Fig. 8 (for additional details, see text).

peptide minimum from 212 to 217 nm suggested a relaxation of the distorted β structure reported in the native enzyme [16], while the shoulders at 208 and 225 nm suggested additional α-helical content; the resolution in this area makes any interpretation highly speculative.

Modification by EPNP

Loss of proteolytic activity in the presence of a 2000-fold molar excess of EPNP would appear to follow pseudo first-order kinetics for the first 48 h (Fig. 8). Loss of activity beyond this point was associated with a disproportionate incorporation of label so that total inactivation required 96 h and was associated with the binding of between 4 and 5 mol of EPNP per mol of enzyme (Fig. 9).

Although the rate of activity loss was not as rapid as in the presence of N_2Ac-Nle-OMe, the half-time of about 14—16 h was still significantly shorter than the value of 20—24 h recorded for penicillopepsin [24] and pepsin [3] presumably due to the lower pH used in the present experiments [24].

Discussion

The progress and the outcome of the reaction of *M. miehei* protease with N_2Ac-Nle-OMe resembled that of other proteases in the sense that the rate of inactivation was copper dependent and the loss of activity was associated with the incorporation of norleucine. However, the kinetics of the reaction are, and were expected to be, complex since the suggested mechanism involves initial binding of a copper-complexed carbene by a carboxylate group with a pK_a of about 4 followed by a transfer to a protonated carboxyl group [2]. In the case of pepsin, there are many possible initial binding sites, since about 10 mol of copper are bound/mol of enzyme at pH 5.6, and several possibilities for the acceptor site since the denaturation of pepsin at neutral pH was associated with the loss of six protons [25,26]. A similar situation exists with respect to *M. miehei* protease. From our titration data, this enzyme has a net negative charge

of about 10 at pH 5.0 suggesting numerous potential copper-binding sites and from a consideration of the pH dependence of thermal denaturation, about six protons are released at pH 5.0 indicating six potential N_2Ac-Nle-OMe-reactive sites [13,27]. Although Lundblad and Stein [2] have reported that the copper-complexed carbene intermediate is responsible for the relative specificity of this reaction, there is no a priori reason why any one of the many negatively charged groups could not bind the intermediate especially since it has been shown that specificity of diazo compounds is not governed by the amino acid residue.

Based on our own results, we would like to suggest an additional role for copper in this reaction. It is possible that a second function of copper is to stablize the structure of the enzyme by forming a square-planar type complex with two carboxyl groups other than those implicated in catalysis [28,1]. Experimental evidence for such a complex has not been presented but it has been demonstrated that the addition of copper brings about an instanteous increase in $[\theta]_{232}$ and $[\theta]_{245}$ (Fig. 4).

In addition we have also found that preparations of N_2Ac-Nle-OMe-inhibited enzyme contain 1 mol of copper which was not removed by extensive dialysis. Furthermore, the H-^{3}H exchange reaction of the modified protein was greatly retarded suggesting a much tighter structure, than in the native enzyme (Fig. 7). On this basis, a second rate-limiting step in the reaction might involve a copper-mediated structural stabilization which facilitates a carbene transfer to the reactive site aspartic acid. The decreased reaction rate observed after 20 min preincubation with copper could then be due to a subsequent loosening of the structure caused by weaker non-specific copper-carboxylate interactions as suggested by the continuing decay of $[\theta]_{232}$ or by cationic competition for the carbene-binding site as suggested by the inhibitory effects of NaCl (Fig. 2). It should be emphasized that without further experimental evidence this "addition role" is speculative at best. At the same time, it is obvious that the function of copper is not restricted to the formation of the carbene intermediate.

M. miehei protease was also progressively inhibited by the addition of increasing amounts of pepstatin; 6 mol of pepstatin/mol of enzyme being required for complete inhibition. In contrast, Kunimoto et al. [23] found that an equimolar concentration of pepstatin was required for total inhibition of pepsin, which is in contrast to the results of a more recent investigation, in which it was concluded that each pepstatin molecule binds two molecules of pepsin [29]. In the present investigation, complete inhibition of *M. miehei* protease was associated with a major conformational change presumably due to the binding of more than 1 mol of pepstatin per mol of enzyme. With respect to this enzyme, the explanation for pepstatin-induced inhibition is not one of simple competitive inhibition [23,29] and is further complicated by the regeneration of activity noted in Fig. 5B.

EPNP inhibition follows a similar course as that reported for penicillopepsin; the disproportionate increase in the incorporation of label after 48 h was presumably due to denaturation and non-specific binding [21]. Since *M. miehei* protease was inhibitable by N_2Ac-Nle-OMe, and pepstatin as well as EPNP, it is obviously a "pepsin-like" acid protease as defined in Introduction. Consequently, the similarities with the glycoproteases of *Sc. lignicolum* noted earlier do not extend to the anomalous reaction with pepsin inhibitors.

Acknowledgements

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