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ANOMALOUS KINETICS OBSERVED IN THE CATALYSIS OF A PEPTIDE SUBSTRATE WITH COLLAGENASE

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SUMMARY

For the catalyzed hydrolysis of the frequently utilized peptide substrate Z-Gly–Pro–Gly–Gly–Pro–Ala with a collagenase, clostridiopeptidase A (EC 3.4.4.19.), an unusual kinetic pattern has been found. The saturation curve displayed an intermediary plateau region which generated two points of inflection. The substrate concentration used was in the range from $0.4 \cdot 10^{-3}$ to $10 \cdot 10^{-3}$ M.

The three criteria for negative cooperativity exhibited by a number of enzymes (Levitski, A. and Koshland, D. E. (1969) *Proc. Natl. Acad. Sci. U.S.*, 62, 1121) have been satisfied with this enzyme preparation. The cooperativity index R_s is greater than 81, the double-reciprocal plot is concave downward, and the Hill coefficient is less than 1.

It is unknown at this time if the described phenomena is related to the subunit structure of this enzyme (mol. wt 110 000) or to the presence of an isoenzyme or other polymorphic forms of the same enzyme. In any case, cooperative effects are indicated in the hydrolysis of this peptide substrate with clostridiopeptidase A.

INTRODUCTION

The kinetics of hydrolysis of small synthetic peptides catalyzed by a collagenase, clostridiopeptidase A (EC 3.4.4.19), from *Clostridium histolyticum* has been studied by two groups of workers. Heyns and Legler[1] first made the observation that the double-reciprocal plot they obtained in their studies of collagenase activity on a synthetic peptide gave a curved line instead of a straight line, such "that the kinetic constants could not be exactly calculated". No further deductions were made in this work. In 1965, Yagisawa *et al.*[2] studied the hydrolysis of the substrate Z-Gly–Pro–Leu–Gly–Pro by the clostridial collagenase. They were able to show that with

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid.

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this substrate the collagenase enzyme followed the Michaelis–Menten kinetics so that the kinetic constants K_m and V were easily calculated. In this investigation, the substrate concentration was varied from $5 \cdot 10^{-3}$ to $40 \cdot 10^{-3}$ M by these workers. The inhibitory effects of several substrate analogues on the hydrloytic reaction of the substrate catalyzed by the enzyme were also tested and evaluated.

The present communication reports an unusual kinetic pattern observed for the hydrolysis of the standard peptide substrate of collagenase. The N-protected peptide of Grassmann and Nordwig[3] Z-Gly–Pro–Gly–Gly–Pro–Ala was used as the substrate in this particular study. Rate studies with this substrate revealed complex kinetics and resemble situations in which enzyme saturation curves possess intermediary plateau regions as has been described recently by Teipel and Koshland[4].

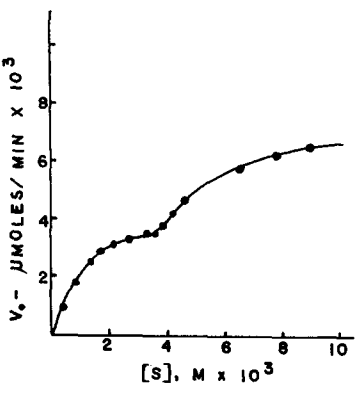
EXPERIMENTAL

This investigation was carried out with a highly purified preparation of clostridiopeptidase A from *C. histolyticum* (CLSPA 9EA 218 units/mg wt, Worthington Biochemical Corp., Freehold, U.S.A.). The substrate Z-Gly–Pro–Gly–Gly–Pro–Ala was purchased from Mann Research Laboratories, New York, U.S.A. and has been shown to be free of contamination as determined by thin-layer chromatography in several solvent systems. In addition, no non-specific cleavage was found with the enzyme preparation as has been reported previously[5]. The rate of hydrolysis of Z-Gly–Pro–Gly–Gly–Pro–Ala in 0.05 M *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES) buffer (pH 7.41) was determined essentially according to the method of Grassmann and Nordwig[3] with the modifications reported by Soberano and Schoellmann[5]. A stock solution of the enzyme was prepared in 0.01 M calcium acetate. The activity, measured as a change in absorbance at 570 nm per min, was shown to be linearly related to the enzyme concentration. The range of substrate concentration used was from $0.4 \cdot 10^{-3}$ to $10 \cdot 10^{-3}$ M. Because of the unusual kinetic pattern which was observed, special care was taken to exclude such trivial explanations as an inhomogeneity of the reaction mixture brought about for instance by some enzyme molecules sticking to the side of the tube. However, treating the reaction vessels and pipettes with dichlorosilane did not prevent the production of the "bumpy" curves.

RESULTS AND DISCUSSION

The velocity *versus* substrate concentration plot (Fig. 1) produced a pronounced deviation from the classical Michaelis–Menten hyperbola. The saturation curve displayed an intermediary plateau region which generated two points of inflection. The original plot was obtained from the average initial velocity of duplicate samples at nine substrate concentrations. The experiment was repeated 3 times, each time the substrate concentrations were selected such that the plateau and the sigmoidal portions of the curve could be confirmed.

When the data of Fig. 1 were plotted in a double-reciprocal plot; the curve which is concave downward as shown in Fig. 2 was obtained. V was calculated to be $14.1 \cdot 10^{-3}$ μmoles Gly–Pro–Ala per min. From the type of curve obtained from the Lineweaver–Burk plot two limiting K_m values could be calculated. The K_m values $9.9 \cdot 10^{-3}$ and $1.9 \cdot 10^{-3}$ M differ from each other by a factor of 5. Conway and Kosh-



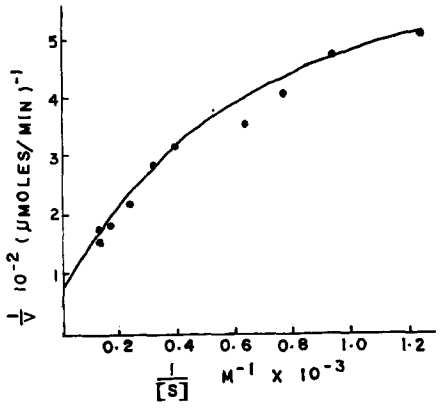


Fig. 1. Michaelis–Menten plot of hydrolysis of Z-Gly–Pro–Gly–Gly–Pro–Ala by collagenase. Initial velocities were determined for the initial 9–30% hydrolysis.

Fig. 2. Lineweaver–Burk plot of data from Fig. 1. Two limiting K_m values can be calculated from this plot which are related to each other by a factor of 5.

land[6] reported that the ratio of the K_m values for enzymes exhibiting the biphasic curve has been observed in the range of between 5 and 12. The ratio of the 90 and 10% velocities, R_s ($S_{0.9}/S_{0.1}$) gave a value greater than 81 ($R_s = 142$)[7]. A Hill plot gave a Hill coefficient of 0.7.

The three criteria for negative cooperativity exhibited by a number of enzymes[8] have, therefore, been satisfied by bacterial collagenase. The cooperativity index R_s is greater than 81, the double reciprocal plot is concave downward and the Hill coefficient is less than 1.

Negative cooperative effects as discussed at length by Levitski and Koshland[8] could be due to a ligand-induced conformation change, that is, a ligand induces a conformation in the proteins which distorts one subunit differently from the other subunits leading to a sequential conformation change. Other possible reasons given to explain the phenomenon cannot be excluded for collagenase at this time until further work can establish the purity of the enzyme preparation and allows us to exclude the presence of isoenzymes or the presence of two or more polymorphic forms of the same enzyme. Furthermore, the question of the subunit structure for this enzyme has to be clarified. Teipel and Koshland[4] have pointed out that a "bumpy curve" will not be produced by an enzyme with only two binding sites and that multisite enzymes will not give this type of curve either when the intrinsic binding or catalytic constants progressively increase, progressively decrease or remain constant with saturation.

A group of Russian workers have proposed that the clostridial enzyme is composed of four subunits[9], whereas Harper *et al.*[10] indicated that collagenase dissociates into two active subunits. Our results seem to favor a four-subunit structure for this enzyme especially if protein homogeneity can be established. Alternately, subunits containing two binding sites each would have to be invoked, not an altogether impossible proposition considering the specificity requirement for efficient substrates, which demands two correctly spaced pyrrolidine residues on both sides of the susceptible peptide bond.

The presence of negative cooperative effects, as defined by Levitski and Koshland[8], is also indicated from preliminary kinetic studies using phenylazobenzyloxycarbonyl-Pro–Leu–Gly–Pro–D-Arg, the collagenase substrate proposed by Wuensch and Heidrich[11].

Ligand binding studies carried out with this enzyme in conjunction with a further elucidation of the structural features of this protein should enable us to interpret our present findings in terms of a possible sequential model of subunit interaction. The functional role which these cooperative phenomena could possibly play in the case of an extracellular bacterial collagenase are totally unknown at the moment. One wonders, however, whether the structure of the native substrate, the collagen triple helix, might have something to do with it.

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