INTRINSIC CATALYTIC ACTIVITY OF THE ZYMOGEN, BOVINE PROCARBOXYPEPTIDASE A. A KINETIC STUDY USING FLUORINE ANALOGUES.

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Summary

Bovine Procarboxypeptidase A (PCPD) has only until recently been considered catalytically inert. PCPD, however, will hydrolyze the amide bond in simple acylated amino acids. Trifluoroacetyl-L-phenylalanine is a very good substrate for the zymogen exhibiting normal Michaelis-Menten kinetics with a Vmax near 2 x 10^3 min^{-1} and a K_m of 2.6 mM. Comparison of the pH-rate profiles for the zymogen-enzyme pair suggest that the same or similar groups are involved in the catalytic process in both proteins further suggesting the pre-existance of a considerable part of the enzyme active site in the zymogen. Moreover, TFAc-D-Phe is a competitive inhibitor of the hydrolysis of TFAc-L-Phe and would appear a suitable analogue to study E (or zymogen)-I interactions by ^{19}F-nmr during activation.

Introduction

It has become increasingly clear that zymogens, enzyme precursors thought to be essentially inert substances, can exhibit intrinsic catalytic (or proteolytic) activity (1). Bovine procarboxypeptidase A seems a clear representative of this phenomenon since the catalytic site has been shown to pre-exist (2) and indeed appears separable from certain features of the binding site. Recently, substrates have been designed specifically for this zymogen and certain halogenated acyl-amino acids do exhibit high turnover rates (3). This communication describes the kinetic properties of trifluoroacetyl-L-phenylalanine (TFAc-L-Phe) which is an excellent substrate for both PCPD and CPD. These and other studies described here should provide a means of investigating the zymogen activation process by means of ^{19}F-NMR and a TFAc-D-Phe inhibitor analogue.

Materials and Methods

Procarboxypeptidase was isolated by the procedure of Behnke et.al. (4)

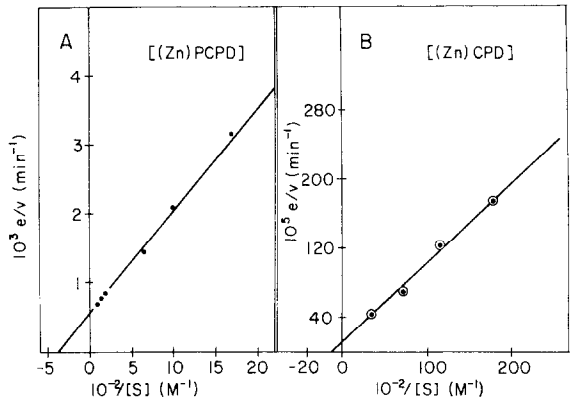

Figure 1. Lineweaver-Burke Plot of the hydrolysis of TFAc-L-Phe by [(Zn)PCPD], (A) ●-●-● and [(Zn)CPD], (B) ◉-◉-◉ . Conditions: 0.05 M Tris HCl, 1.0 M NaCl, pH 7.5, 25°. Assay conc. of [(Zn) PCPD] = 2 x 10^{-8}M; [(Zn)CPD] = 1 x 10^{-9} M.

and was found to be homogeneous by ultracentrifugation, electrophoresis and Sephadex gel-filtration on G-150. The zymogen displayed only the very low activities (2.5%) observed using normal carboxypeptidase substrates such as carbobenzoxyglycyl phenylalanine. No evidence of spontaneous activation was detected prior to or subsequent to the kinetic analyses reported here. Peptidase assays were performed according to Auld et.al. (5) using an automated Technicon system. Trifluoroacetyl L- or D-phenylalanine were prepared by treatment of D- or L-phe (Sigma) with anhydrous trifluoroacetic anhydride at 0°. Crystallization occurred spontaneously on addition of water and the derivative was twice recrystallized from a water-ethanol mixture.

Results and Discussion

Both PCPD and CPD exhibit high turnover rates for TFAc-L-Phe, Vmax being 1.7 x 10^3 and 2.0 x 10^4 min^{-1}, respectively. The Lineweaver-Burk plots (figure 1) for the hydrolysis of TFAcPhe using initial rate data are linear over at least a tenfold concentration range. This linearity is surprising considering the marked deviations exhibitied by other small molecular substrates such as CGPhe and HPLA, the latter a corresponding ester substrate. Indeed, TFAc-L-Phe behaves similarly to the tripeptide substrates, CGG-L-Phe and BzGG-LOPhe. Km values for the zymogen and enzyme are similar, (1.7

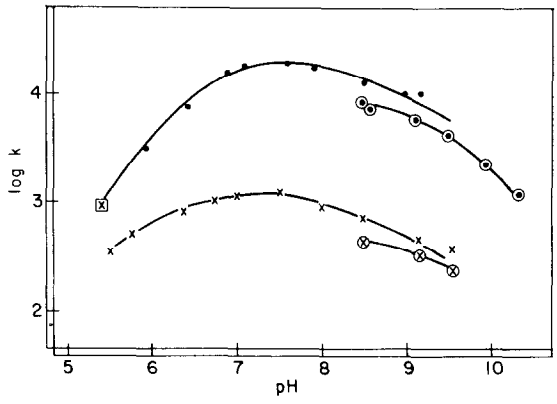

Figure 2. The pH dependence of log k for [(Zn) PCPD], x-x-x, and [(Zn)CPD], ●-●-●. Concentration of the substrate, TFAc-L-Phe, was fixed at 0.01M (10 fold above Km for the enzyme) except in the alkaline portion where concentration of 0.005 M was used due to high background absorbance; ⊗-⊗-⊗ , indicates 0.005 M TFAc-L-Phe for [(Zn)PCPD]; ◉-◉-◉ , for [(Zn)CPD]. [X] designates the addition of excess zinc (10^{-4} M). Conditions: 1.0M NaCl, 0.05 M Tris, Mes, Hepes or carb-bicarb, buffers, 25°.

and 2.6 mM, respectively) though the Vmax values differ by more than a factor of 10. The variation of log (k) as a function of pH for carboxypeptidase and procarboxypeptidase over the pH range 5-10 is shown in Figure 2. The curves are very similar and indicate the same approximate (apparent) pK values for the ionizing groups affecting catalysis.*

Notable are the similarities in these values for <u>both</u> proteins which provides further support for the apparent pre-existance of catalytic groups in the zymogen precursor. Normalization of the pH-log rate profiles for the zymogen-enzyme pair show some deviations in the low pH region but calculation of the apparent pK values reveals little difference. In each case, ionization of groups with approximate pK values of 6.3 ± 0.10 and 8.8 ± 0.10 are indicated. No effect on activity was observed by adding excess zinc ions in the low pH region where competition with protons might be expected.

In view of the obvious similarities of the zymogen-enzyme pair, mechan-

* The curve is a more complex function than indicated and has been resolved for the enzyme by plotting log (Kcat/Km) versus pH (5). This shows the bell-shaped curve to be a composite of a variable Kcat in one region and a variable Km in another.

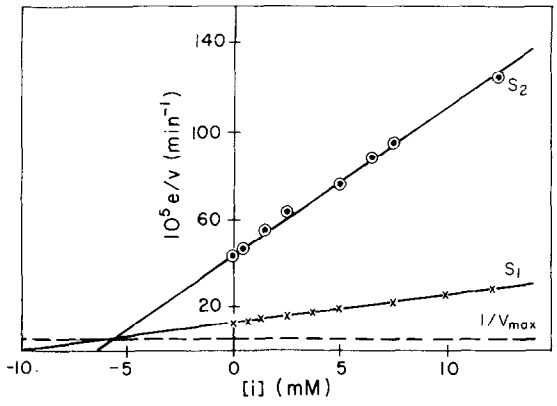

Figure 3. Dixon plot of the inhibition of [(Zn)CPD] hydrolysis of TFAc-L-Phe by TFAc-D-Phe. S_1 (x-x-x) = 1.22 x 10^{-3} M TFAc-L-Phe (≈ Km); S_2 = (⊙-⊙-⊙) 2.5 x 10^{-4} M TFAc-L-Phe (4 fold below Km). Conditions: 0.5 m Tris Hcl, 1.0 M NaCl, pH 7.5, 25°. A similar inhibition is observed for the zymogen activity.

istic schemes developed for the enzyme could similarly be developed for the zymogen in the absence of activation, though the absolute rates for zymogen hydrolysis are considerably lower and remain to be explained.

In further support of a pre-existing catalytic site, the substrate specificity requirements of the zymogen were investigated. TFAc-L-gly, TFAc-L-Phe-NH_2 and TFAc-D-Phe are not hydrolyzed by either the zymogen or CPD. TFAc-D-Phe is, in fact, a competitive inhibitor of the hydrolysis of TFAc-L-Phe for both PCPD and CPD (figure 3). This latter observation provides new opportunities for studying the pre-existing catalytic site in the zymogen and the activation process itself. Inhibitor interactions with the potential active site could be explored by ^{19}F-NMR using TFAc-D-Phe chemical shift data and T_1 relaxations. Moreover, TFAc-D-Phe could be lengthened sequentially with glycyl (or alanyl) residues to probe other possible catalytic determinants not revealed until after activation. This should present a more detailed view of the process of zymogen activation in PCPD and the molecular requirements for catalytic expression in this molecule. Finally, a sensitive assay system has been developed for the zymogen *per se*, thus providing a gauge of pre-activation catalytic function

and the structure-function relationships found therein. From the specificity requirements, this activity is clearly related to eventual carboxypeptidase function and may provide an important clue to the precise activation mechanism.

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