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Substrate Enantiomers. Modifiers of Carboxypeptidase A Activity†

Louis G. Lange, III, David S. Auld, and James F. Riordan*

ABSTRACT: The dual specificity of carboxypeptidase A toward ester and peptide substrates was studied by employing substrate enantiomers. The present investigation demonstrates that BzGly-D-OPhe inhibits the hydrolysis of BzGly-L-OPhe. Moreover, dependent on the particular substrate employed, this, and other substrate enantiomers, can activate, inhibit, or leave unaltered the rate of carboxypeptidase-catalyzed hydrolysis. The modes of inhibition have been characterized kinetically, and changes in the circular dichroic spectrum of carboxypeptidase, when labeled with a conformational probe, imply alterations in protein structure consequent to enantiomer binding. The results confirm previous postulates that the catalytic mechanisms of dipeptide and ester hydrolysis by carboxypeptidase include multiple productive and nonproductive enzyme–substrate complexes.

Differences between ester and peptide hydrolysis have long been observed for both native and various modified carboxypeptidases[1] (Vallee *et al.*, 1970). Several years ago a dual-site model, whose basic tenet was multiple nonidentical but overlapping binding sites for esters and peptides, was proposed to account for a number of the observed kinetic phenomena (Vallee *et al.*, 1968). The model suggested that carboxypeptidase could recognize differences between ester and peptide linkages. While this functional group discrimination had been observed only with substrates, it seemed likely that it might also occur with substrate enantiomers. Hence, BzGly-D-OPhe and BzGly-D-Phe, the optical isomers of the most commonly employed substrate pair, were synthesized and their association with carboxypeptidase was examined.

Materials and Methods

Substrates. The synthesis of BzGlyGly-L-Phe has been described (Auld and Vallee, 1970) and that of BzGlyGly-L-OLeu will be described elsewhere (B. Holmquist and D. S. Auld, in preparation). BzGly-L-Phe, from Yeda Chemical Co., and BzGly-L-OPhe, obtained from Fox Chemical Co. and recrystallized from dry acetone, mp 74–75°, were suitable for use.

BzGly-D-OPhe was synthesized from D-Phe according to the method of McClure (1966) and yielded white crystals which were recrystallized from dry acetone and dried *in vacuo* over

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received December 10, 1973.* This work was supported by Grants-in-Aid GM-15003 and GM-02123 from the National Institutes of Health, of the Department of Health, Education and Welfare.

[1] Abbreviations used are: Bz, benzoyl; OPhe, phenyllactate; OLeu, β-isopropyllactate; carboxypeptidase refers to carboxypeptidase A throughout.

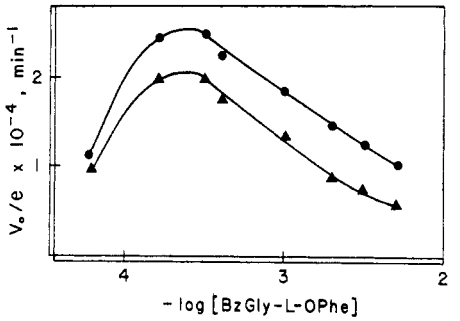

FIGURE 1: Velocity *vs.* substrate concentration profiles for the carboxypeptidase catalyzed hydrolyses of BzGly-L-OPhe (●) and BzGly-DL-OPhe (▲).

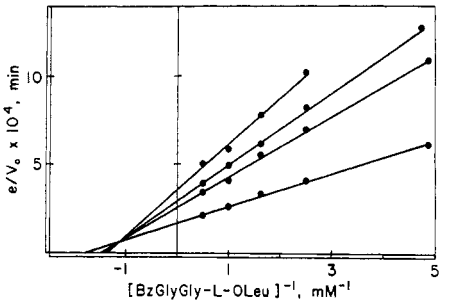

FIGURE 3: Lineweaver–Burk plots for inhibition of BzGlyGly-L-OLeu hydrolysis by BzGly-D-Phe. The slopes of the lines increase as the inhibitor concentration increases from zero to 1.0, 2.0, and 6.0×10^{-3} M, respectively.

P_2O_5, mp 74–75°. *Anal.* Calcd: C, 61.92; H, 4.61; N, 4.01. Found: C, 61.79; H, 4.72; N, 4.05. Thin-layer chromatography on silica gel developed with methanol–water (1:1) identified a single component. Optical rotation at 10 mg/ml in deionized, distilled water gave $[\alpha]_D^{21} = +5.28°$. The product was completely resistant to hydrolysis by carboxypeptidase under the assay conditions used in this study.

BzGly-D-Phe was synthesized from D-Phe according to the procedure of Anderson *et al.* (1964) and afforded a white solid, mp 141–142°, after several recrystallizations from ethyl acetate–petroleum ether (bp 30–60°). *Anal.* Calcd: C, 66.24; H, 5.55; N, 8.58. Found: C, 66.09; H, 5.67; N, 8.43. Optical rotation at 18.0 mg/ml in absolute ethanol gave $[\alpha]_D^{20} = -38.0°$. The product was not hydrolyzed by carboxypeptidase under standard conditions.

All buffer solutions were extracted with 0.01% dithizone in CCl_4, and other precautions, previously described, were taken to minimize contamination by adventitious metal ions (Thiers, 1957).

Enzymes. Bovine pancreatic carboxypeptidase A prepared according to the procedure of Anson (1937) was obtained from Worthington Biochemical Corp. as an aqueous suspension of crystals stored under toluene. The crystals were washed three times with deionized, distilled water and dissolved in 3 ml of 0.01 M Tris–3 M NaCl at pH 7.5. A 0.05-ml aliquot of this stock solution was diluted into 2.0 ml of 0.01 M Tris–1 M NaCl (pH 7.5) and the protein concentration ($\sim 5 \times 10^{-6}$ M) was determined from absorbance at 278 nm using a Zeiss PMQ II spectrophotometer. A molar absorptivity of 6.42×10^4 M^{-1} cm^{-1} was employed for the native enzyme (Simpson *et al.*, 1963). Further dilutions were made, as appropriate, two minutes before each assay point in the kinetic studies.

Arsanilazotyrosyl-248 carboxypeptidase was prepared and its circular dichroic spectrum determined as previously reported (Johansen and Vallee, 1973).

Esterase activity was determined by pH titration of the protons released on hydrolysis using a Radiometer pH-Stat and recorder (Snoke *et al.*, 1948). All titrations were carried out on either 2 or 5 ml of substrate dissolved in 0.07 M NaCl at pH 7.5 using 2 mM NaOH as the titrant. A nitrogen atmosphere was established and the reaction vessel was thermostatted at 25 ± 0.2°. With enzyme concentrations of 10^{-8}–10^{-10} M, initial rates were linear for hydrolysis of 10% of the substrate.

Peptidase activity was measured at 25 ± 0.2° in 0.05 M Tris–1 M NaCl (pH 7.5) by a modification of the ninhydrin method (Snoke and Neurath, 1949) employing a Technicon AutoAnalyzer (Auld and Vallee, 1970).

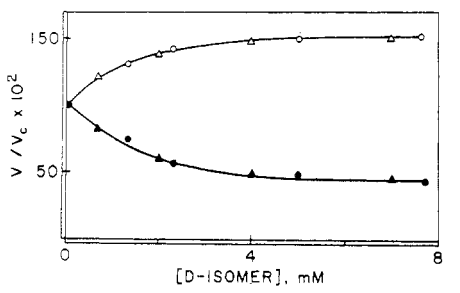

FIGURE 2: Activation of BzGly-L-Phe hydrolysis [1×10^{-3} M] (open symbols) and inhibition of BzGly-L-OPhe hydrolysis [5×10^{-3} M] (closed symbols) as a function of the concentration of BzGly-D-Phe (△,▲) and BzGly-D-OPhe (○,●).

Results

Inhibition of BzGly-L-OPhe Hydrolysis. The carboxypeptidase-catalyzed hydrolysis of BzGly-L-OPhe exhibits kinetic anomalies over a wide substrate concentration range, but throughout it is more rapid than that of the racemic mixture, BzGly-DL-OPhe (Figure 1). This suggests that the D enantiomer might act as an inhibitor. Indeed, at a substrate concentration of 5×10^{-3} M, the rate of BzGly-L-OPhe hydrolysis progressively decreases as the concentration of BzGly-D-OPhe increases up to 7.5×10^{-3} M (Figure 2). Above this concentration, no further effect on activity is observed. The peptide analog, BzGly-D-Phe, also inhibits the hydrolysis of BzGly-L-OPhe and to the same degree as the D ester (Figure 2). No attempts were made, however, to characterize the mode of inhibition by either one of these two D enantiomers using BzGly-L-OPhe as substrate, since the hydrolysis of this ester proceeds with quite complex kinetics.

Activation of BzGly-L-Phe Hydrolysis. In contrast to their effect on the hydrolysis of BzGly-L-OPhe, both BzGly-D-OPhe and BzGly-D-Phe activate the carboxypeptidase catalyzed hydrolysis of the N-substituted dipeptide BzGly-L-Phe (Figure 2). The degree of activation is virtually the same for the two substrate enantiomers, being 159 and 147%, respectively, relative to the control.

BzGlyGly-L-Phe Hydrolysis. Although the D enantiomers increase the rate of hydrolysis of the substituted dipeptide, they do not activate the hydrolysis of the corresponding tripeptide, BzGlyGly-L-Phe. Activities obtained at a tripeptide substrate concentration of 2.75×10^{-4} M, an $[S]/K_m$ value of 0.25, in the presence of 5×10^{-3} M BzGly-D-OPhe or BzGly-D-Phe are 93 and 92% of the control, respectively.

Inhibition of BzGlyGly-L-OLeu Hydrolysis. Carboxypeptidase hydrolyzes the ester BzGlyGly-L-OLeu according to Michaelis–Menten kinetics; there is neither substrate activation nor inhibition (B. Holmquist and D. S. Auld, in preparation). Hence, this substrate was employed to establish the

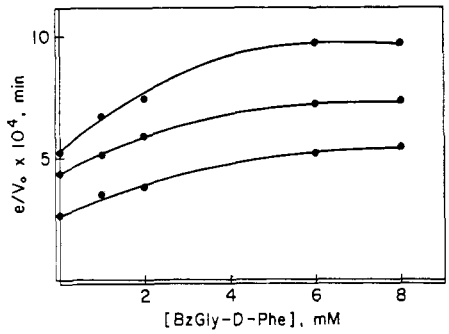

FIGURE 4: Dixon plots for inhibition of BzGlyGly-L-OLeu hydrolysis by BzGly-D-Phe. Concentrations of substrate were 4.0, 6.0, and 20.0 $\times$ 10^{-4} M, the top, middle, and bottom curves, respectively.

modes of inhibition of ester hydrolysis by the D enantiomers. Lineweaver–Burk plots with the concentration of BzGly-D-Phe varying from 0 to 6 mM result in a pattern of intersecting lines indicative of mixed inhibition (Figure 3), but the effect on k_{cat} is predominant. Dixon plots of the inhibition of BzGlyGly-L-OLeu hydrolysis by the D peptide demonstrate that inhibition reaches a finite limit at inhibitor concentrations above 6 mM and [S]/K_m values of 1–5 (Figure 4). This type of behavior fits the description of mixed inhibition which is predominantly noncompetitive in nature (Dixon and Webb, 1964).

In contrast to the results with the D-peptide, Lineweaver–Burk plots at concentrations of the ester, BzGly-D-OPhe, varying from 0 to 10 mM demonstrate an apparent increase in K_m while k_{cat} remains constant (Figure 5). Furthermore, varying the concentration of D ester at a fixed substrate concentration demonstrates a progressive increase of inhibition that does not reach a plateau (Figure 6). The type of behavior observed in both the Lineweaver–Burk and Dixon plots is typical of fully competitive inhibitors (Dixon and Webb, 1964). Both the D ester and D peptide have K_i's in the range 3–5 $\times$ 10^{-3} M.

Effect on the Circular Dichroic Spectrum of Arsanilazotyrosyl-248 Carboxypeptidase. The circular dichroic spectrum of arsanilazotyrosyl-248 carboxypeptidase, known to reflect the microenvironment of the active center of the enzyme (Johansen and Vallee, 1971, 1973), was examined in the presence of BzGly-D-OPhe or BzGly-D-Phe. On addition of either one of the D enantiomers at 5 $\times$ 10^{-3} M, the magnitude of the negative ellipticity band at 510 nm is reduced from 38,500 to approximately 30,000 (deg cm²)/dmol.

Discussion

Previous studies of the behavior of substrate enantiomers as modifiers of carboxypeptidase activity have been limited generally to comparisons of the hydrolytic rate of the racemic substrate mixture with that of the L isomer alone. Some of the first experiments reported (Snoke *et al.*, 1948; Snoke and Neurath, 1949) showed that both the L and DL forms of BzGly-OPhe exhibit the same type of complex kinetic behavior. This led to the conclusion that the presence of the D isomer was not the cause of the observed substrate inhibition. On the basis of these results it has since been held (McClure *et al.* 1964; Bender *et al.*, 1965; Whitaker *et al.*, 1966; Davies *et al.*, 1968b; Bunting and Murphy, 1972) that BzGly-D-OPhe does not inhibit BzGly-L-OPhe hydrolysis. It should be noted, however, that turnover numbers for the hydrolyses of the L and DL ester substrates have not been compared at similar substrate concentrations. Further, systematic inhibition studies

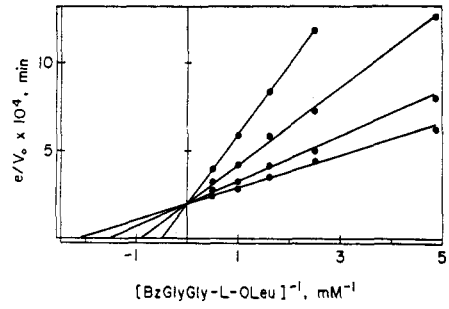

FIGURE 5: Lineweaver–Burk plots for inhibition of BzGlyGly-L-OLeu hydrolysis by BzGly-D-OPhe. The slopes of the lines increase as the inhibitor concentration increases from zero to 2.0, 8.0, and 10.0 $\times$ 10^{-3} M, respectively.

using the D isomer have never been performed. A k_{cat} value for BzGly-L-OPhe hydrolysis has been determined, and was found to coincide with values reported for that of BzGly-DL-OPhe (Whitaker *et al.*, 1966). However, the complexity of the kinetics of the hydrolysis of this ester renders comparisons of such kinetic constants tenuous (Davies *et al.*, 1968b). On the other hand, more recent work (Schechter and Zazepizki, 1971) demonstrated that the D enantiomers of a wide variety of peptides bind to carboxypeptidase and, in fact, activate dipeptide hydrolysis by affecting K_m, in qualitative accord with similar observations using hydrolysis products (Davies *et al.*, 1968a).

The present studies indicate unequivocally that the rate of the carboxypeptidase-catalyzed hydrolysis of BzGly-DL-OPhe is less than that of BzGly-L-OPhe (Figure 1). The difference in rates does not appear to be related to a change in the mode or extent of substrate inhibition but rather to the presence of the D isomer. Detailed analysis revealed a progressive decrease in the rate of hydrolysis of BzGly-L-OPhe on adding increasing amounts of BzGly-D-OPhe (Figure 2). However, in view of the persistent anomalous kinetics, a definite assignment of the type of inhibition is not feasible.

Since carboxypeptidase can apparently discriminate between ester and peptide substrates, the effect of the D peptide, BzGly-D-Phe, on the hydrolysis of BzGly-L-OPhe was also examined. Under equivalent conditions, both D enantiomers reduced the rate of hydrolysis of BzGly-L-OPhe and virtually to the same extent (Figure 2).

The depsipeptide analog, BzGlyGly-L-OLeu, whose hydrolysis obeys Michaelis–Menten kinetics, was chosen to quantitate the esterase inhibition. With this substrate BzGly-D-OPhe acts as a fully competitive inhibitor (Figures 5 and 6) while, on the other hand, BzGly-D-Phe acts as a mixed, predominantly noncompetitive inhibitor (Figures 3 and 4).

The capacity of carboxypeptidase to discriminate between esters and peptides when bound productively is emphasized

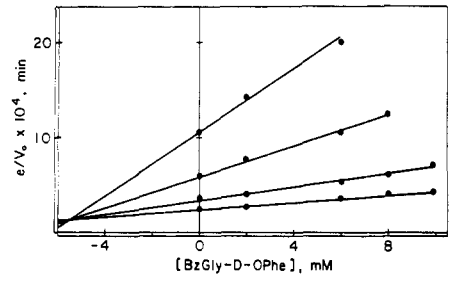

FIGURE 6: Dixon plots for the inhibition of BzGlyGly-L-OLeu hydrolysis by BzGly-D-OPhe. Lines of increasing slope were obtained with substrate concentrations of 1.0, 2.0, 10.0, and 20.0 $\times$ 10^{-4} M, respectively.

futher by the effects of the D enantiomers on the hydrolysis of BzGly-L-Phe. In marked contrast to their behavior toward BzGly-L-OPhe, both D enantiomers *activate* the hydrolysis of the benzoyl dipeptide (Figure 2). These effects are similar to those observed for a large number of various compounds which have the capacity of increasing the peptidase activity while inhibiting esterase activity (Davies *et al.*, 1968a). This behavior has been interpreted in terms of the dual-site model (Vallee *et al.*, 1968). Such activators are thought to partially obstruct the binding site for ester hydrolysis while simultaneously preventing "wrong-way" binding to the binding site for peptide hydrolysis. The former would lead to inhibition and the latter to apparent activation.

The hydrolysis of N-substituted tripeptides has been shown to be devoid of self-activation (Auld and Vallee, 1970). According to the dual-site model, the interaction of these larger peptide substrates places greater constraints on the enzyme–substrate complexes formed, thereby eliminating some nonproductive binding modes. The D enantiomers, similar to other activators of dipeptide hydrolysis, fail to affect the hydrolysis of BzGlyGly-L-Phe appreciably.

These results indicate that the D isomers can serve to distinguish between esters and peptides when bound productively. Thus, both the D ester and the D peptide inhibit the hydrolysis of BzGly-L-OPhe but activate that of BzGly-L-Phe. Moreover, neither activate the hydrolysis of the tripeptide, BzGlyGly-L-Phe, but both inhibit that of the ester, BzGlyGly-L-OLeu.

From the similarity of the actions of the D ester and the D peptide, it can be said that carboxypeptidase probably does not distinguish between esters and peptides when bound nonproductively. Although there appear to be some differences in the modes of inhibition of BzGlyGly-L-OLeu hydrolysis by the D enantiomers, in general, they affect activity similarly, both in kind and in degree.

In addition, they bring about similar effects on the conformation of the enzyme as judged by changes in the circular dichroic spectrum of arsanilazotyrosyl-248 carboxypeptidase. This chemically modified enzyme retains both esterase and peptidase activities and displays extrinsic Cotton effects at 335 nm (π–π^*), 425 nm (n–π^*), and 510 nm (zinc–arsanilazotyrosyl-248 complex) which are maximal at pH 8.2 and have been used to probe the conformation of carboxypeptidase (Johansen and Vallee, 1973). Since the azochromophore reports changes in protein conformation near the active site, the reduction in magnitude of the circular dichroic trough at 510 nm upon addition of either BzGly-D-OPhe or BzGly-D-Phe suggests that in each case binding induces analogous conformational changes in the enzyme. It is further suggested that their effect on activity may in part be mediated by an alteration of the microenvironment of the active site.

The conclusion that nonproductively bound esters and peptides bind similarly while productively bound esters and peptides bind differently is consistent with recent postulates concerning the molecular origin of such discrimination (Riordan, 1973). Productive peptide binding is thought to involve interaction of the free carboxyl group of the substrate with an arginyl residue of carboxypeptidase, orientation of the amido hydrogen toward Tyr-248 and polarization of the scissile carbonyl bond by the zinc ion. In contrast, arginyl residues do not appear necessary for binding the carboxyl group of ester substrates. Kinetic studies of metal-substituted carboxypeptidases have recently demonstrated that the metal functions primarily in the catalytic step of peptide hydrolysis but primarily in the binding step of ester hydrolysis (Auld and Holmquist, 1973). This would be expected if ester binding involved a carboxyl–metal rather than a carboxyl–arginine interaction. It is not unexpected then that such a three-dimensional system, able to discriminate between L esters and L peptides, would be unable to discriminate between esters and peptides of completely different three-dimensional conformation such as the D esters and D peptides.

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