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# Tetrazole analogs of amino acids as constituents of modifiers of carboxypeptidase A catalysis

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The action of substrate analogs containing the tetrazolyl group instead of the C-terminal carboxy group on the peptidase activity of carboxypeptidase A is studied. The analogs compete with the substrate for the secondary binding site thus showing activation phenomena.

Carboxypeptidase A; Substrate analog; Competitive activator; Secondary binding site; Amino acid analog

## 1. INTRODUCTION

The 5-substituted tetrazolyl group has some structural features in common with the carboxy group [1]. The acidity of the tetrazole nucleus corresponds with that of a carboxy group in peptides [2] and tetrazolate and carboxylate anions both have planar structures. To study the role of free carboxy groups in biologically active systems several tetrazole analogs of amino acids and biologically active peptides were synthesized and structure-function relationships in enzymatic [3–5] and hormonal [6] systems were investigated.

For these studies carboxypeptidase A (EC 3.4.17.1) should be an interesting example, since it needs a free carboxylate group in the C-terminal position of a peptide substrate which binds by two hydrogen bonds to Arg-145 of the active site of the enzyme [7].

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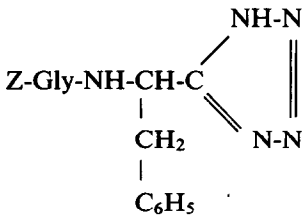
*Abbreviations:* Z, benzyloxycarbonyl; H-Gly-T, 5-aminomethyl-1(2)H-tetrazole; H-Phe-T, L-5-$\alpha$-aminophenylethyl-1(2)H-tetrazole; T, 5-substituted tetrazolyl group; ONp, *p*-nitrophenyl ester

We have found that Z-Gly-Phe-T (I), the tetrazole analog of the typical substrate of carboxypeptidase A (Z-Gly-Phe-OH), is not hydrolyzed by the enzyme but acts as a modifier of the enzyme catalysis.




(I)

Here, the mode of action of derivatives of tetrazole analogs of amino acids was tested for their capacity to modify the kinetics of the hydrolysis of Z-Gly-Phe-OH by carboxypeptidase A. Z-Gly-Phe-OH itself and Z-Gly-OH are known to cause stimulation of the peptidase activity of the enzyme [8,9]. The mechanism of this acceleration of carboxypeptidase A activity has been described [7,10].

## 2. MATERIALS AND METHODS

Z-Gly-OH, Z-Gly-Phe-OH [11] and Z-Gly-T [12] were prepared in our laboratories. Z-Gly-Phe-

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T was synthesized from Z-Gly-ONp and L-H-Phe-T according to [13]: m.p. 140–142°C; $[\alpha]_D^{20}$ = −21.8° ($c$ = 1, MeOH). TLC, $R_f$ 0.34 ($CHCl_3$:EtOH, 3:1, v/v); $R_f$ 0.47 ($CHCl_3$:MeOH:AcOH, 18:1:1, v/v) on silica gel 60 plates (Merck). For $C_{19}H_{20}N_6O_3$, found: C 60.1%, H 5.1%, N 21.9%; calculated: C 60.0%, H 5.3%, N 22.1%.

Carboxypeptidase A (bovine pancreas) was purchased from Serva (Heidelberg). Enzyme stock solutions in 2.36 mol/l LiCl and 1 mmol/l Na-veronal-HCl, pH 7.6, were prepared according to [10]. Kinetic measurements were made at 25°C, pH 7.6, 0.6 mol/l LiCl, 22.5 mmol/l Na-veronal-HCl as described in [10]. The liberated phenylalanine from substrate hydrolysis was determined by the colorimetric ninhydrin procedure of [14]. All experiments were run in duplicate. Due to substrate activation the Lineweaver-Burk plot was not a straight line (curves A and B in fig.2). For the evaluation of the kinetic parameters a computer program [15] was employed.

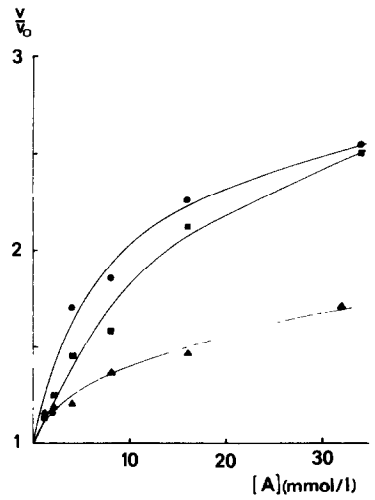



Fig.1. Acceleration of carboxypeptidase A activity at constant substrate concentration (Z-Gly-Phe-OH, 1.25 mmol/l). Activators (A): (●) Z-Gly-OH, (▲) Z-Gly-T, (■) Z-Gly-Phe-T, $v$ and $v_0$ represent velocities in the presence and absence of activator, respectively.

## 3. RESULTS AND DISCUSSION

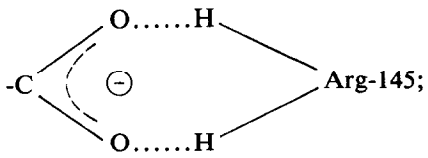
In several experiments (prolonged incubation, high enzyme concentrations) it was shown that Z-Gly-Phe-T was not split by carboxypeptidase A. The substrate analog remained unchanged as judged by chromatographic methods. To study the potential inhibitory effect of the substrate analog we added this compound to incubation mixtures of carboxypeptidase A and Z-Gly-Phe-OH. Surprisingly we observed an activation phenomenon.

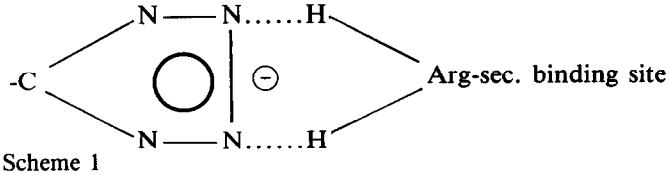
The effects of different concentrations of Z-Gly-OH, Z-Gly-T and Z-Gly-Phe-T on the hydrolysis of Z-Gly-Phe-OH are shown in fig.1 and agree well with similar experiments by other authors [10] using Z-amino acids and -peptides as modifiers which were not split by the enzyme.

Kinetic measurements of the activation phenomenon revealed that the tetrazole analogs compete with the activating substrate molecule in a secondary binding site (Arg-127, Arg-71, [7]) (fig.2).

The influence of high concentrations (30 mmol/l) of the analog I on the kinetic parameters of the enzymatic hydrolysis of Z-Gly-Phe-OH results in a decrease in $K_m$ by a factor of 2.6 (table 1) indicating the formation of an enzyme-modifier complex which has a higher affinity for the substrate than the free enzyme. The catalytic capacity of this complex ($k_{cat}$) is increased nearly 2-fold as compared with the free enzyme.

Besides the high capacity of the tetrazolate ion to form hydrogen bonds [16] our experiments demonstrate that the substrate analog cannot bind to Arg-145. This may be due to the different shapes of the tetrazolate and carboxylate ions respectively in hydrogen bonding (scheme 1).




Scheme 1

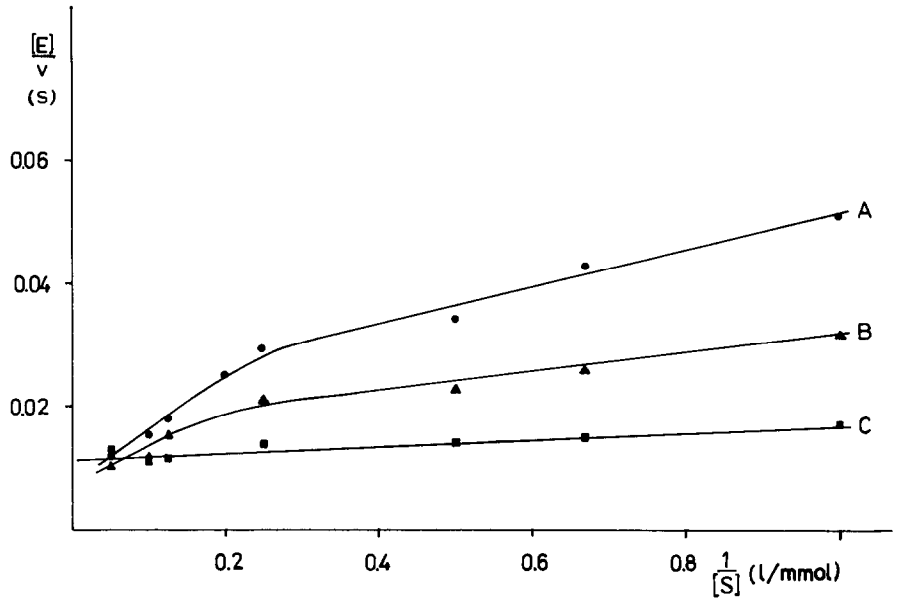



Fig.2. Acceleration of carboxypeptidase A activity at constant activator concentration. Lineweaver-Burk plot for the hydrolysis of Z-Gly-Phe-OH in the absence (●) and presence of Z-Gly-Phe-T at 6 mmol/l (▲) and 30 mmol/l (■).

Table 1

Kinetic data for the hydrolysis of Z-Gly-Phe-OH (1–20 mmol/l) by carboxypeptidase A at pH 7.6 in the presence of high concentrations of Z-Gly-Phe-T

| $K_m$ (mmol·l$^{-1}$) | $k_{cat}$ ($s^{-1}$) | $C$ (l·mol$^{-1}$·$s^{-1}$) | $K_{m_o}/K_m$ | $k_{cat}/k_{cat_o}$ | $C/C_o$ |
|---|---|---|---|---|---|
| 0.47 ± 0.092 | 86.0 ± 2.52 | 183000 | 2.6 | 1.9 | 5.0 |

$K_{m_o}$ (1.21 ± 0.215 mmol·l$^{-1}$), $k_{cat_o}$ (44.2 ± 2.95 $s^{-1}$) and $C_o = k_{cat_o}/K_{m_o}$ (36530 l·mol$^{-1}$·$s^{-1}$) represent data (mean values ± SD) in the absence of modifier at low concentrations of Z-Gly-Phe-OH (1–4 mmol/l, no substrate activation observed, see curve A in fig.2). $1/K_m$, $k_{cat}$ and $C$ represent data in the presence of Z-Gly-Phe-T (30 mmol/l). The difference of the kinetic parameters is significant within a confidence interval of 2.4 × SD (98.36%)

The strong chelating properties of the tetrazole groups [17] seem not to interfere with the Zn atom of the active center of carboxypeptidase A for even at high concentrations of the tetrazole analogs no inhibition of the peptidase activity was observed.

The results can be understood best if it is assumed that the tetrazole analogs bind to the secondary binding site [7] thus forming an enzyme-modifier complex which has higher activity than the free enzyme (table 1).

## ACKNOWLEDGEMENTS

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