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A Thioamide Substrate of Carboxypeptidase A†

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ABSTRACT: Carbobenzoxythioglycyl-L-phenylalanine [CbzNHCH$_2$C(=S)Phe, Z-Gly^S-Phe] was synthesized as a thioamide analogue of Z-Gly-Phe, a known substrate of carboxypeptidase A (CPA). By use of a ninhydrin-based assay and Z-Gly-Gly-Phe as the substrate, Z-Gly^S-Phe was shown to be a weak, competitive inhibitor of CPA (K_i = 1.4 mM). The L isomer (but not the D) of Z-Gly^S-Phe proved to be a substrate for CPA (K_m = 1.1 mM and k_{cat} = 5.3 s^{-1} at pH 7.5), binding with comparable affinity to, but hydrolyzing at 10% the rate of, the oxo analogue Z-Gly-Phe. The CPA-catalyzed hydrolysis of Z-Gly^S-Phe was shown to involve only C–N bond cleavage, to give carbobenzoxythioglycine and phenylalanine.

Carboxypeptidase A (CPA)[1] has been the subject of intense investigation for many years, and more is known of its properties and behavior than any other zinc-containing peptidase (Lipscomb, 1980, and references cited therein; Kaiser & Kaiser, 1972; Hartsuck & Lipscomb, 1971; Pĕtra, 1970; Vallee & Riordan, 1968). Crystal structures have been determined for inhibitor complexes as well as for the free enzyme (Hartsuck & Lipscomb, 1971; Lipscomb, 1974; Rees & Lipscomb, 1980; Rees et al., 1980), and its activity with peptide and ester substrates, at various pHs and with various metals in place of zinc, has been extensively explored (Auld & Holmquist, 1974; Turk & Marshall, 1975; Makinen et al., 1979; Auld & Vallee, 1970a,b, 1971; Pĕtra, 1970; Coleman & Vallee, 1960). There remains, however, a degree of uncertainty about the actual mechanism by which a peptide

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[1] Abbreviations: CPA, carboxypeptidase A; Z-Gly, carbobenzoxyglycine; Z-Gly^S, carbobenzoxythioglycine; Z-Gly-Phe, carbobenzoxyglycyl-L-phenylalanine; Z-Gly^S-Phe, carbobenzoxythioglycylphenylalanine (unless otherwise indicated, this refers to the L isomer); Z-Gly-Gly-Phe, carbobenzoxyglycylglycyl-L-phenylalanine; Phe, phenylalanine; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; DCC, dicyclohexylcarbodiimide; IR, infrared spectrum; ^{1}H NMR, proton magnetic resonance spectrum; UV, ultraviolet spectrum; MS, mass spectrum.

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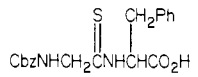
substrate is cleaved by the enzyme, and further investigation is warranted (Lipscomb, 1980; Makinen et al., 1979; Breslow & Wernick, 1977).

Recently we reported a phosphorus-containing dipeptide analogue which is a potent inhibitor of CPA (Jacobsen & Bartlett, 1981). The most tightly bound inhibitor of the enzyme so far discovered, however, is 2-benzyl-3-mercaptopropanoic acid [Ondetti et al., 1979; see also Holmquist & Vallee (1979)], which suggests that there is an especially favorable interaction between the zinc atom in the enzyme active site and a sulfur ligand. With this stimulation, we reasoned that the study of a thioamide analogue of a typical CPA substrate could provide valuable information, irrespective of whether the compound was an inhibitor or a substrate. Consequently, we synthesized carbobenzoxythioglycylphenylalanine (Z-Gly^S-Phe) and studied its interaction with



Z-Gly^S-Phe

CPA. We report here our finding that Z-Gly^S-Phe is in fact a substrate for the peptidase.

Experimental Procedures

Materials. Z-Gly, Z-Gly-Phe, Z-Gly-Gly-Phe, L-phenylalanine methyl ester hydrochloride, and ninhydrin reagent solution were purchased from Sigma Chemical Co. and used directly. Bovine CPA was obtained from Worthington or Calbiochem and dissolved in 10% LiCl as a stock solution (Decker, 1977). The buffer solution employed throughout was pH 7.5 25 mM Tris-HCl containing 0.5 M NaCl.

Carbobenzoxythioglycylphenylalanine (Z-Gly^S-Phe). The dicyclohexylcarbodiimide-induced coupling of carbobenzoxyglycine and L-phenylalanine methyl ester afforded carbobenzoxyglycyl-L-phenylalanine methyl ester as a colorless oil after HPLC purification (1:4 ethyl acetate–chloroform/silica gel): $[\alpha]_D$ +13.3° (*c* 0.94 in ethanol); IR ($CHCl_3$) 3430, 1735, 1683 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.1 (d, 2, J = 5.5 Hz), 3.6 (s, 3), 3.8 (d, 2, J = 5.5 Hz), 4.8 (m, 1), 5.1 (s, 2), 5.5 (m, 1), 6.6 (br d, 1), 7.1 (m, 5), 7.3 (s, 5). A sample of racemic material (obtained from DCC-induced esterification of Z-Gly-Phe) was obtained in crystalline form: mp 75–76 °C (ether).

The thioamide was formed on treatment of 1.88 g (5.3 mmol) of Z-Gly-Phe methyl ester in 50 mL of CH_2Cl_2 with 2.38 g (5.3 mmol) of P_4S_{10} and 3 mL (21.4 mmol) of triethylamine at 21 °C for 72 h. The product was isolated by washing the mixture twice with 2 N HCl and 3 times with water, drying ($MgSO_4$), and evaporation of the solvent. The product (1.84 g, 94% crude yield) was obtained as an oil which was clean enough to be used without further purification: IR (film) 3330, 1720 cm^{-1}; 60-MHz ^{1}H NMR ($CDCl_3$) δ 3.2 (AB of ABX, 2, apparent J = 2 and 5 Hz), 3.7 (s, 3), 4.2 (d, 2, J = 6 Hz), 5.1 (s, 2), 5.4 (m, 2), 7.1 (m, 5), 7.3 (s, 5), 8.3 (br s, 1); MS m/z 386.1295 (expected for $C_{20}H_{22}N_2O_4S$: 386.1300).

The methyl ester of Z-Gly^S-Phe (390 mg, 1.0 mmol) was hydrolyzed with 1.1 mmol of NaOH in 12 mL of 4:1 acetone–water at 21 °C for 20 h. The mixture was evaporated, diluted with water (25 mL), washed with ether (3 × 25 mL), acidified to pH 1 with 2 N HCl, and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layer was washed with water (2 × 50 mL), dried (Na_2SO_4), filtered, and evaporated to give 0.36 g of a yellow oil. After purification by preparative TLC (250:25:1 $CHCl_3$–CH_3OH–CH_3CO_2H/silica gel) and evaporation from ether, 244 mg (65% yield) of Z-Gly^S-Phe was obtained as a free-flowing, but clearly amorphous, white powder: $[\alpha]_D$ +85.2° (*c* 3.23 in ethanol); IR ($CHCl_3$) 3300, 2960, 1720, 1500, 1210 cm^{-1}; 250-MHz ^{1}H NMR (Na salt in CD_3OD) δ 3.15, 3.43, and 5.01 (ABX, J_{AB} = 13.6 Hz, $J_{AX} \approx J_{BX} \approx$ 5.2 Hz), 4.05 and 4.21 (AB, 2, J_{AB} = 17.3 Hz), 5.07 (s, 2), 7.15 (br s, 5), 7.32 (br s, 5); ^{13}C NMR (Na salt in H_2O) δ 36.3, 51.6, 61.3, 67.2, 126.7, 127.6, 128.3, 128.5, 128.6, 129.0, 135.9, 136.9, 157.6, 175.9, 198.9; MS m/z 354 ($M^+ - H_2O$), 91 (base); UV max (methanol) 264 nm (ϵ 11 900).

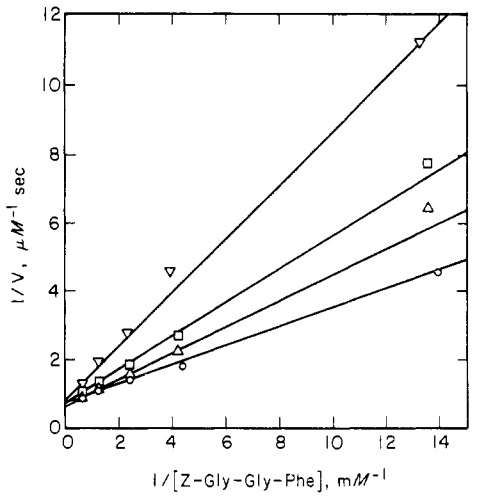


FIGURE 1: Double-reciprocal plot of the rate of CPA-catalyzed hydrolysis of Z-Gly-Gly-Phe at pH 7.5, 25.0 °C, as a function of substrate concentration in the absence (○) and presence of Z-Gly^S-Phe [(△) = 0.59 mM; (□) = 1.14 mM; (▽) = 2.34 mM]. Total enzyme concentration is 1.5 × 10^{-8} M. In the absence of Z-Gly^S-Phe, K_m = 0.37 mM and k_{cat} = 95 s^{-1}.

Acid-catalyzed hydrolysis of this material (4 N HCl at 80 °C for 5 h) and reverse-phase HPLC analysis of the resulting mixture with a proline/cupric ion mobile phase modifier (Gil-Av et al., 1980) revealed the presence of less than 10% of D-phenylalanine, indicating that only minor racemization had occurred during the thionation and alkaline hydrolysis steps.

A racemic sample of Z-Gly^S-Phe was prepared by using similar thionation and hydrolysis procedures starting with racemic Z-Gly-Phe methyl ester. In contrast to the optically active form, this material was obtained in crystalline form: mp 150–151 °C ($CHCl_3$–hexane). Anal. Calcd for $C_{19}H_{20}N_2O_4S$: C, 61.27; H, 5.41; N, 7.52; S, 8.61. Found: C, 61.20; H, 5.39; N, 7.54; S, 8.49.

Carbobenzoxythioglycine (Z-Gly^S). The thio acid was prepared from carbobenzoxyglycine by the method of Bartmann & Wieland (1956): mp 92.5–93 °C ($CHCl_3$) (lit. mp 93–95 °C); UV (buffer) 246 nm (ϵ 7400).

Z-Gly^S-Phe as an Inhibitor of CPA. The improved ninhydrin assay method (Moore, 1968; Elkins-Kaufman & Neurath, 1949; Schwert, 1948) was used to determine the rate of hydrolysis of Z-Gly-Gly-Phe in the presence of varying amounts of Z-Gly^S-Phe at 25 °C (see Figure 1). Although data are presented in double-reciprocal form, the kinetic parameters were determined by the direct linear method (Eisenthal & Cornish-Bowden, 1974). The average concentration over the course of an incubation was used in order to correct for substrate depletion (Segel, 1975).

Z-Gly^S-Phe as a Substrate of CPA. The rate of CPA-catalyzed hydrolysis of Z-Gly^S-Phe at 25 °C was followed spectrophotometrically, monitoring the change in absorbance at 238 nm ($\Delta\epsilon$ = +3900) (see Figure 2). As above, the kinetic parameters were determined by the direct linear method.

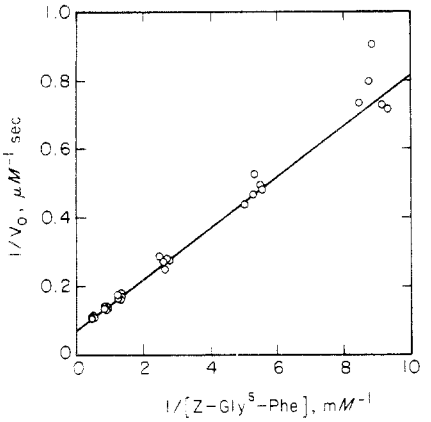


FIGURE 2: Double-reciprocal plot of the rate of CPA-catalyzed hydrolysis of Z-Gly^S-Phe at pH 7.5, 25.0 °C. Each cluster of points represents five duplicate assays. Total enzyme concentration is 2.75 × 10^{-6} M.

Table I: UV Spectral Determination of Enzymatic Hydrolysis Products

	λ_{max} (nm)	ϵ
Z-Gly + Phe + Na_2S[a,b]	228	4600
Z-Gly^S + Phe[b,c]	247	7400
end point of CPA-catalyzed hydrolysis of Z-Gly^S-Phe[d]	247	7200

[a] [Z-Gly] = [Phe] = [Na_2S] = 6.3 × 10^{-5} M in buffer, against a buffer reference. [b] The presence of 10^{-5} M CPA in both the sample and reference had no influence on the UV spectrum. [c] [Z-Gly^S] = [Phe] = 6.2 × 10^{-5} M in buffer, against a buffer reference. [d] [Z-Gly^S-Phe] = 5.6 × 10^{-5} M at the start of reaction; [CPA] = 1.2 × 10^{-5} M in both the sample and reference.

Determination of Enzymatic Hydrolysis Products. The course of the enzymatic cleavage reaction was determined by comparing the UV spectrum of a reaction mixture which had gone to completion with spectra of equimolar mixtures of phenylalanine with Z-Gly^S or Z-Gly and sodium sulfide (Table I).

Results and Discussion

Z-Gly^S-Phe is a weak, competitive inhibitor of CPA, with K_i = 1.4 mM (Figure 1). Because of the high extinction coefficient of the thioamide moiety (ϵ_{264} = 11 900) and the fact that high concentrations are required to observe appreciable inhibition, the convenient spectrophotometric assay of Z-Gly-Gly-Phe hydrolysis could not be employed (Auld & Vallee, 1970a). A ninhydrin-based assay was therefore used.

Z-Gly^S-Phe is a substrate for CPA, with K_m = 1.1 mM and k_{cat} = 5.3 s^{-1} (Figure 2). In this case, because of the large absorbance change during the course of the reaction ($\Delta\epsilon_{267}$ = −10 100: $\Delta\epsilon_{238}$ = +3900), a straightforward spectrophotometric assay was possible. We chose to monitor the change in absorbance at 238 nm because the absolute absorbance at this wavelength is lower than at 267 nm. This CPA-catalyzed hydrolysis reaction is specific for the L enantiomer of Z-Gly^S-Phe, as demonstrated by the fact that the reaction with racemic material stops at 50% completion.

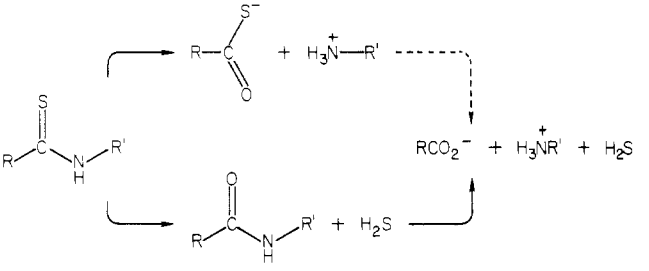
The similarity of K_i (1.4 mM) and K_m (1.1 mM) for Z-Gly^S-Phe suggests that this compound occupies the same binding site for both inhibition and hydrolysis. More interesting is the fact that Z-Gly^S-Phe is bound to the enzyme with comparable affinity to the oxoamide Z-Gly-Phe itself: a K_m value of 2.0 mM has been reported under similar conditions (Neurath et al., 1968; Pétra, 1970). The replacement of the carbonyl group with a thiocarbonyl moiety does not appreciably alter the enzyme-binding characteristics of the dipeptide, in spite of the considerably longer bond length [1.7 Å for C=S vs. 1.2 Å for C=O (Duus, 1979; Gordon & Ford, 1972)], or any special sulfur–zinc interaction. This result may simply mean that in the Michaelis complex amide substrates do not bond to the zinc atom through the carbonyl oxygen (Lipscomb, 1980; Turk & Marshall, 1975; Auld & Holmquist, 1974).

It has been suggested that such carbonyl–zinc coordination requires twisting about the scissile bond (Lipscomb, 1980; Cleland, 1977; Mock, 1975); this is resisted by the double bond character of the peptide linkage. Thioamides have an even higher C–N rotation barrier (Löwenstein et al., 1964; Walter & Maerten, 1968) and would therefore also be expected to bind in the Michaelis complex without coordination to the metal.

Not surprisingly, the rate of CPA-catalyzed hydrolysis of Z-Gly^S-Phe (k_{cat} = 5.3 s^{-1}) is slower than that for the oxo analogue Z-Gly-Phe [k_{cat} = 55 s^{-1} at similar substrate concentrations (Neurath et al., 1968; Pétra, 1970)]. Nonetheless, it is significant and specific for the L isomer. None of the anomalous kinetic behavior of the oxo analogue was observed with the thioamide, although the substrate concentration range we investigated (0.1–2.0 mM) is considerably below that at which such effects have been observed (Davies et al., 1968).

At the time we carried out this work, no peptidase-catalyzed cleavage of a thioamide had been reported. Previously reported sulfur-containing peptidase substrate analogues had been confined to ester derivatives: a *thiono* ester [RC(=S)OR′] has been shown to acylate the cysteine protease papain (Lowe & Williams, 1965); for CPA itself, a *thiolo* ester [RC(=O)SR′] substrate is known (Suh & Kaiser, 1975). After submission of our original manuscript, Mock et al. (1981) reported a study of a different thioamide (benzoyl-Gly-Gly^S-Phe) as a substrate for both native (zinc) and cadmium CPA. Interestingly, they found that the longer thioamide is an exceedingly poor substrate for native CPA ($k_{cat} \approx 0.3\%$ that of the oxo analogue), although the cadmium enzyme is more effective. The reason for the large difference in susceptibility to enzymatic hydrolysis between Z-Gly^S-Phe and benzoyl-Gly-Gly^S-Phe is not immediately apparent.

Two reaction pathways for cleavage of the thioamide linkage



by CPA are theoretically possible: direct cleavage to give the thio acid (with or without subsequent desulfurization to the carboxylic acid) or dethionation followed by rapid cleavage of the resulting oxoamide. Simultaneous occurrence of the two pathways has been observed for both acid- and base-catalyzed hydrolysis of thioamides (Seydel, 1966; Peeters & de Ranter, 1974; Hall & Satchell, 1974).

After complete hydrolysis of Z-Gly^S-Phe by CPA, the UV spectrum is essentially the same as that of a mixture of phenylalanine and the thio acid Z-Gly^S and significantly different from that of phenylalanine, the carboxylic acid Z-Gly, and sodium sulfide (see Table I). The enzymatic hydrolysis of Z-Gly^S-Phe therefore involves only C–N bond cleavage and no loss of sulfur. The thio acid Z-Gly^S itself was tested as an inhibitor of CPA but showed no effect up to 0.64 mM.

Although this initial observation of thioamide reactivity with CPA does not itself address the question of the enzyme mechanism, it opens the door to a number of other explorations. The thioamide and thio acid moieties are obtained with only a single-atom replacement in a "normal" substrate or product and therefore represent a minimal deviation in structure. These groups are strongly UV absorbing, however, which will facilitate both low-temperature and fast-reaction kinetic studies. The importance of the thiocarbonyl–metal interaction is amenable to investigation by replacing the zinc atom in CPA with more thiophilic metals (Jaffe & Cohn, 1978, 1979) as recently shown (Mock et al., 1981) or by spectral studies with more chromophoric metals (Holmquist & Vallee, 1979). We are currently exploring some of these and related studies.

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