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Properties of Analogues of an Intermediate in the Process of Mechanism-Based Inactivation of Carboxypeptidase A

Soumitra S. Ghosh,[a] Srikanth Dakoji,[b] Yasuhiro Tanaka,[b] Young J. Cho[b] and Shahriar Mobashery[b,*]

[a]*Applied Genetics, San Diego, CA 92121, U.S.A.*
[b]*Department of Chemistry, Wayne State University, Detroit, MI 48202, U.S.A.*

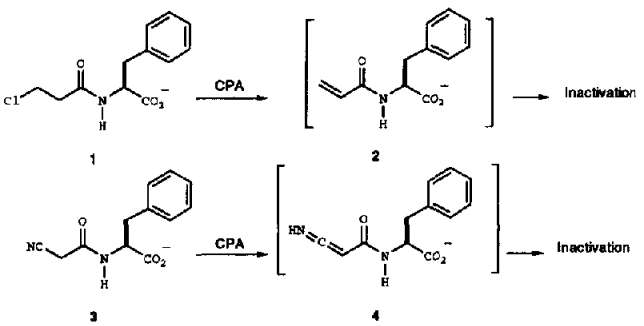
Abstract—Carboxypeptidase A (CPA), and other zinc-dependent proteases, facilitate an α deprotonation of judiciously designed ketones and amides. This adventitious reaction has been used in the development of effective mechanism-based inactivators for this family of enzymes. *N*-Acryloyl-L-phenylalanine, an intermediate in the process of mechanism-based inactivation of CPA by *N*-(3-chloropropionyl)-L-phenylalanine, was shown to be an affinity inactivator, but also a very poor substrate for the enzyme. Similarly, *O*-(acryloyl)-L-3-phenyllactate was shown to be both an affinity inactivator and a poor substrate for CPA. However, consistent with the trend established with other ester and amide substrates for CPA, *O*-(acryloyl)-L-3-phenyllactate is a better substrate than *N*-acryloyl-L-phenylalanine. *N*-(Propiolyl)-L-phenylalanine served only as a poor substrate for the enzyme. To gain insight into enzyme inactivation and the unexpected poor turnover of these molecules, molecular modeling of these compounds with the crystal structure of CPA was carried out. These analyses suggested that the smaller size of these molecules permits a binding mode which is somewhat different in the active site than with typical larger substrates, such that the transition-state species for hydrolysis is not greatly stabilized by the enzyme. The slow turnover of these species, along with their specific binding interactions with the enzyme active site have implications for the inactivation chemistry of CPA and other zinc proteases by this family of mechanism-based inactivators. Copyright © 1996 Elsevier Science Ltd

Introduction

We have reported over the past few years on the development of a class of novel peptide mechanism-based inactivators for metalloproteases.[1–5] An important step in the mechanism of action of these inactivators is an enzyme-mediated deprotonation reaction in suitably designed molecules. We exploited the deprotonation chemistry in unmasking electrophilic species from the inactivators, which upon trapping of an active-site nucleophilic amino-acid residue result in irreversible inactivation of the enzyme. For example, we have shown that *N*-(3-chloropropionyl)-L-phenylalanine (**1**) and *N*-(cyanoacetyl)-L-phenylalanine (**3**) inactivate carboxypeptidase A (CPA), a zinc protease, irreversibly.[1] We had suggested that the requisite deprotonation reaction on **1** is ensued by β-elimination of the chloride to give *N*-(acryloyl)-L-phenylalanine (**2**), whereas the same deprotonation for **3** would result in the formation of the ketenimine species **4**. Species **2** and **4** were offered as the putative electrophilic entities responsible for the irreversible enzyme inactivation. We report herein on the nature of the stereoelectronic interactions of compound **2** and its analogues with the CPA active site which sheds further light on some unusual properties of these molecules.

Results and Discussion

Compound **2** is relatively stable in aqueous solution, and was synthesized for study as an affinity inactivator



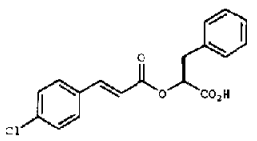
1 2 3 4

for CPA.[1] This compound, as was expected, inactivated CPA in a time-dependent manner. However, we had reported earlier that compound **2** was not a substrate for CPA.[1] This earlier observation was puzzling to us in light of the fact that the compound is an amide and that substituted acryloyl derivatives are generally good substrates for CPA.[6,7] For example, *O*-(*trans*-*p*-chlorocinnamoyl)-L-3-phenyllactate (**5**) is an excellent substrate for CPA (Table 1).[6] Further evaluation of **2**

Table 1. Kinetic parameters for interactions with carboxypeptidase A

Compounds	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	k_{inact} (min^{-1})	K_I (mM)
2	2.4×10^{-3}	2.0	1.2	0.04	1.4
6	0.3	3.3	86.7	0.02	15.0
7	1.6×10^{-2}	0.4	40.8	—	—
5[6a]	13.8	0.03	5.2×10^{5}	—	—
glycyl-L-tyrosine[8]	1.5×10^{-2}	0.7	21.4	—	—

as a substrate for CPA revealed that it is indeed a substrate for CPA at concentrations below 1.0 mM (Table 1; $k_{cat} = 2.4 \times 10^{-3}$ s^{-1}, $K_m = 2.0$ mM, $k_{cat}/K_m = 1.2$ M^{-1} s^{-1}), but it is one of the worst known substrates for the enzyme. In terms of k_{cat}/K_m, this compound is a worse substrate than glycyl-L-tyrosine—a known poor substrate for CPA[8]—by a factor of 18-fold (Table 1).



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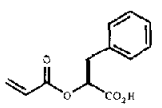
We prepared compound **6**, the ester analogue for **2**. This ester was also both a substrate and an affinity inactivator for CPA (Table 1). Consistent with earlier reported trends that ester substrates are hydrolysed more efficiently than the corresponding amide substrates by CPA, ester **6** is turned over 72-fold more favorably than the amide **2**, as judged from the values for k_{cat}/K_m (Table 1).The effect of enhanced turnover of the ester stems essentially entirely from an increase in k_{cat}.



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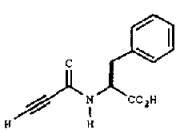
In contrast to compounds **2** and **6**, both of which are substrates and affinity inactivators, *N*-(propiolyl)-L-phenylalanine (**7**) was only a substrate for CPA. As judged by the k_{cat}/K_m values, CPA turns over this compound 34-fold better than **2**. Table 1 also tabulates the kinetic parameters for glycyl-L-tyrosine and ester **5**.



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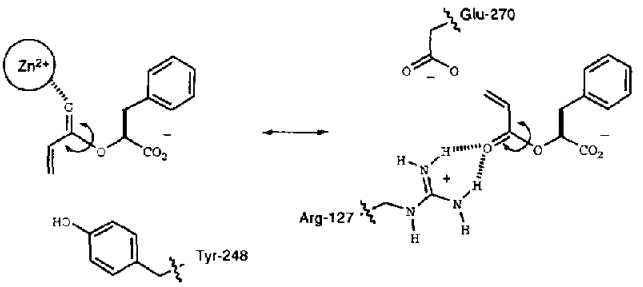
The fact that compounds **2**, **6**, and **7** are such poor substrates is both puzzling and intriguing. Furthermore, the observation that the propiolyl group in **7** does not serve as a Michael acceptor in inactivation of the enzyme indicates that the β-carbon of the propiolyl function is not poised for trapping of the active-site nucleophilic residue. Prompted by these findings, we have carried out molecular modeling of **2** and its analogues in the CPA active site to gain insight on their unusual reactivity.

Catalysis by CPA has been suggested to involve polarization of the scissile carbonyl of the substrate either by coordination of the carbonyl oxygen by the active-site



Scheme 1.

zinc ion or through hydrogen bonding interaction with the side chain of Arg-127.[9,10] The issue of which of the two modes of binding accounts for the activation of substrates for the hydrolytic reaction is currently a subject of much debate, therefore we considered both possibilities in our modeling. Whereas some subtle conformational differences were discernible between the two cases, subsequent to energy minimization, remarkably similar complexes resulted from either binding variant for compounds **2** and **6**, studied for these analyses.

It would appear that a mere rotation, as depicted for **6** in Scheme 1, may allow for coordination of the ester carbonyl by either the zinc ion or the side chain of Arg-127. Subsequent to minimization of energy, the position of the phenyl group, anchored in the hydrophobic pocket of the active site, did not change and distances to active-site residues such as Arg-145 and Asn-144, remain within ±0.1 Å for the corresponding distances in the two complexes. However, the modes of binding significantly influenced the orientation of the β-carbon of the acryloyl moiety within the active site. Thus, if carbonyl coordination were with Arg-127, then Glu-270 would be the likely residue which would be trapped, whereas if carbonyl coordination were with the zinc ion, Tyr-248 could be the likely residue to undergo modification. The picture for the binding of compound **2** to CPA was similar to that for **6**.

The reason that compounds **2**, **6**, and **7** are such poor substrates for CPA was at first unclear. The electronic effects of the acryloyl or propionyl groups cannot account as the origin of the rate retardation, since the cinnamoyl ester **5**,[6] for example, is a good substrate. Furthermore, we observe that some other small substrates for CPA, such as *N*-acetyl-L-phenylalanine, *N*-acetyl-L-tryptophan, and *N*-formyl-L-phenylalanine are also poor substrates for CPA.[11]

In order to explore any potential differences in the active-site binding of the smaller molecules versus the cinnamoyl ester **5**, we generated an energy-minimized complex for the active-site binding of **5**. The binding of the cinnamoyl moiety in compound **5** is different from the acryloyl groups in **2** and **6**. The positions of the side chains of residues Tyr-198 and Phe-279 provide a physical barrier for the binding of the cinnamoyl group in the active site in an extended conformation, thereby forcing it to bend towards the active-site opening. This

is quite analogous to the binding of the phosphonate inhibitor **8**—a transition-state analogue—to the active site, as seen in the crystal structure.[12] In contrast, such steric encumbrance is not observed for **2** or **6** because of the small size of the acryloyl group; the acryloyl group was somewhat ensconced in the active site for these compounds. Figure 1 shows a view of the binding mode for **6**, and compares it to the crystal structure for the transition-state analogue **8** in the enzyme active site.[12] The difference in the active-site binding for **6** and **5** (or **6** and **8**, as shown in Figure 1) stems from steric encumbrance, which should account for the significant difference in their respective rates for turnover.[13] It would appear that the transition-state species for hydrolysis of the smaller substrate(s) is not as well stabilized by the enzyme because of the alternative binding mode of these molecules.

We have also noted that acylation of Tyr-248 of CPA prevented enzyme inactivation by **2**. While Tyr-248 cannot be ruled out as the active-site nucleophilic residue, there is ample precedent suggesting that CPA acetylation may merely affect productive binding of **2** in the active site for enzyme inhibition. Thus, site-directed mutagenesis and crystallographic studies have implicated Tyr-248 in hydrogen-bond donation to substrates, an interaction that would be abolished by acetylation.[9,14] To our knowledge, there are no known substrate-based inactivators for CPA that modify Tyr-248.[15] Each of the inactivators **9**,[16] **10**,[17] and **11**[18] are known to modify Glu-270 covalently. It is likely that compounds **2**, and **6** also alkylate Glu-270 as well, hence the binding mode which brings the β-carbon of the acryloyl moiety near the Glu-270 carboxylate (Scheme 1) may be the likely binding interaction which accounts for the inactivation chemistry.

Finally, we had also considered an additional possibility for the mechanism of the inactivation chemistry. In light of the fact that the acryloyl group—either in **2** or **6**—is not known to be a very good Michael acceptor, we speculated whether a π-d orbital interaction between the olefin moiety of the acryloyl group and the zinc ion, as depicted in Scheme 2, may account for activation of the acryloyl group toward trapping of the nucleophilic amino acid. Assuming that the carboxylate and the benzyl groups in compounds **2** and **6** bind in the enzyme active site in a comparable manner to that seen in the reported crystal structures of inhibitors in the active site of CPA, this possibility was precluded because of the steric constraints of the active site.

In conclusion, the slow process for turnover of these small substrates by CPA suggests that the attenuated rate of substrate hydrolysis indeed may have a bearing on the inactivation chemistry. A molecule such as **2**, when generated within the active site, would not readily undergo hydrolysis, permitting for longer longevity of the enzyme-bound species. This longevity would in turn make possible the slower process of active-site modification of the enzyme and the attendant loss of activity.

Experimental

Kinetic measurements were carried out on a Perkin–Elmer Lambda 3B or Hewlett–Packard 452 diode array instrument. Carboxypeptidase A (EC 3.4.17.1, Type 1 from Bovine Pancreas) was purchased from the Sigma Chemical Co. HPLC analysis was carried out on a Perkin–Elmer Series 410-BIO system. The effluent was monitored by a Perkin–Elmer variable-wavelength

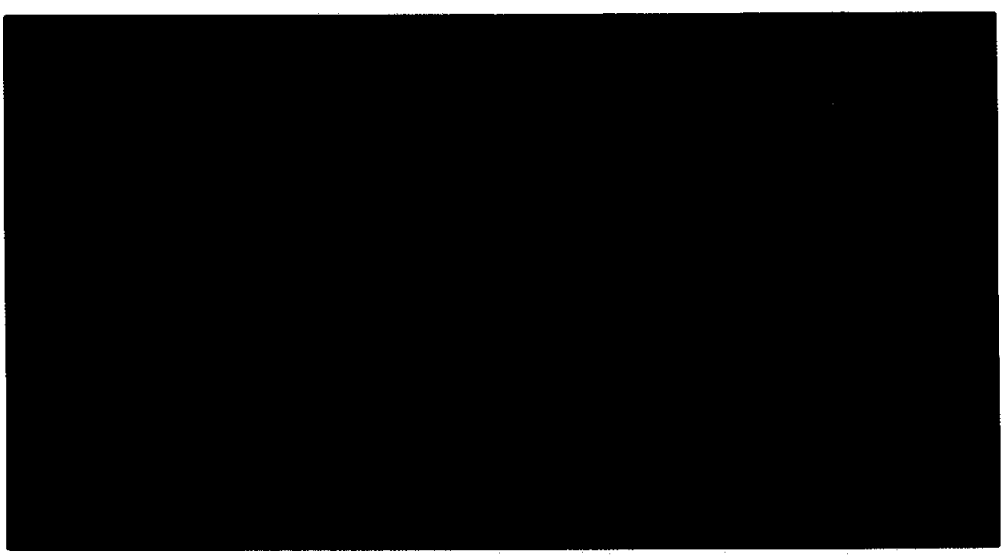
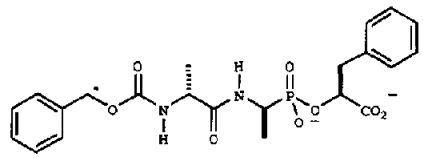


Figure 1. Stereoview of the energy-minimized complex of compound **6** in the active site of CPA. Compound **6** is shown in white with its oxygen atoms depicted in red; the ester carbonyl is coordinated to the Arg-127 side chain and the active-site zinc ion is shown as a yellow sphere. The crystal structure of inhibitor **8** is superimposed in the same space, showing one phosphonate oxygen coordinated to the active-site metal. A number of the active-site amino-acid residues are shown in cyan. Steric interactions with amino-acid residues Phe-279 and Try-198 direct the backbone of inhibitor **8** in the direction of the active-site opening. The smaller size of compound **6** permits the enzyme to bind the compound in its stretched form, a binding mode that is not seen for inhibitor **8**.



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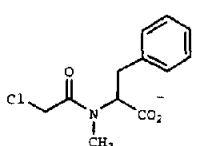
LC-95 detector. ^1H and ^{13}C NMR spectra were recorded on a Nicolet QE-300 or Varian-500 spectrometer. IR and MS were recorded on Nicolet 20 DX and Kratos MS 80 RFA spectrometers, respectively. Enzyme activity was measured by monitoring hydrolysis of a chromogenic ester *O*-(*trans*-*p*-chlorocinnamoyl)-L-3-phenyllactate at 320 nm.[6a] A typical assay (1.0 mL) consisted of 500 μM of the substrate ester and 20 nM enzyme in 10 mM MOPS, 0.5 M NaCl, pH 7.0. Acetylated CPA was prepared by the literature procedure.[19] The crystal structure 6CPA, obtained from the Brookhaven National Laboratory, was used in modeling.

Solvation of enzyme–inhibitor complexes

Crystal coordinates for CPA (6CPA, Protein Data Bank, Brookhaven National Laboratory) were used in the three-dimensional modeling and energy minimization. The existing phosphonate inhibitor in the active site of CPA in the crystal coordinates was eliminated. The structures of **2**, **6**, and **7** were docked into the active site individually. A total of 148 crystallographic water molecules were retained for the subsequent minimization, and the active-site-bound inactivator was then covered by the addition of Monte Carlo water molecules in each case. In addition, the areas of the enzyme which were covered by the original phosphonate inhibitor in the crystal coordinates, but not by the docked molecule, were covered by the Monte Carlo water molecules as well.

Energy minimization of the complexes

A dielectric constant of 1.0 was used for the calculations. The hydrogen atoms were added in the calculated positions and atomic charges were computed by the method of Gasteiger–Hückel.[20] Energy minimization was carried out in three steps: (1) the docked molecule and water molecules were allowed to move, (2) the docked molecule, water molecules, and the protein backbone were allowed to move, and (3) finally, the entire complex was allowed to minimize without any constraints. The energy minimization in a radius of 15 Å from the active site in each stage was continued until the change in energy was less than 0.001 kcal/mol/Å between iterations.



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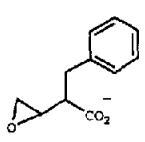

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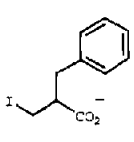

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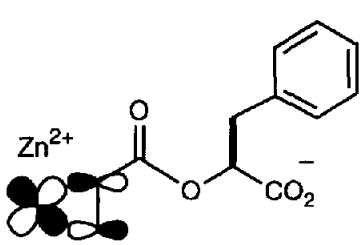

Scheme 2.

Kinetic analyses

Inactivation experiments were carried out with compound **6**, and were attempted with compounds **7**. In a typical inactivation experiment, a portion of the stock solution of the inactivator in 1,4-dioxane was added to the enzyme (0.1 μM), giving a final concentration of 10% dioxane in 100 mM MOPS supplemented with 0.5 M NaCl, pH 7.0. Aliquots (10 μL) of the mixture were diluted individually into assay mixtures (990 μL) at various time intervals and the remaining enzyme activity was monitored immediately. Analysis of the data was carried out according to Ghosh et al.[1]

A protection experiment was carried out with **6** as follows. The enzyme (0.1 μM) was incubated with (*R,S*)-benzylsuccinic acid (1 μM) for 10 min at room temperature. Subsequently, compound **6** was added to the mixture to give a final concentration of 15 mM. The enzyme activity was monitored over time, as described above.

Kinetic parameters for hydrolysis of **2** and **7** were determined according to the method of Stein and Moore.[21] Typically, six to seven different concentrations of the substrate (0.1–1.0 mM) were incubated with CPA (2.7 μM) in 250 mM MOPS, 0.5 M NaCl, pH 7.0 for 20 min at room temperature. A 250-μM portion of each mixture was analysed with ninhydrin for the presence of phenylalanine according to Stein and Moore. Each determination was carried out in duplicate.

It was possible to measure the initial rates for hydrolysis of the ester substrate **6** by spectrophotometric means. The rate for hydrolysis of the first 5% of the ester was monitored at 236 nm ($\Delta\varepsilon_{236}=248\ M^{-1}cm^{-1}$). Six different concentrations of the substrate (0.6–0.9 mM) were used in the assays in 250 mM MOPS, 0.5 M NaCl, pH 7.0, each determined in duplicate, with 2.7 μM enzyme. We attempted to determine the kinetic parameters for hydrolysis of **2** by this method as well; however, due to much poorer turnover of **2**, this experiment was technically not possible.

***O*-(Acryloyl)-L-3-phenyllactic acid (6).** *N*-Methylmorpholine (218 μL, 2.0 mmol) was added to a solution of L-3-phenyllactic acid (166 mg, 1.0 mmol) in 1.0 mL of dry THF under an atmosphere of argon, and the mixture was stirred for 5 min. Acryloyl chloride (81 μL,

1.0 mmol) was added to the mixture in a dropwise fashion, and the solution was stirred in the dark for 16 h. Subsequently, the mixture was concentrated in vacuo and the residual oil was taken up in ethyl acetate (≈40 mL). The organic layer was washed with 1 N HCl (2×), water, and was then dried over anhyd Na_2SO_4. The solvent was evaporated to dryness, followed by purification by flash chromatography on silica gel (3:1, ethyl acetate:hexane containing 1% acetic acid) to provide 138 mg of the title compound (63% yield). For the kinetic analysis, a portion of the product was further purified by preparative TLC using 85:10:5 chloroform:methanol:acetic acid (R_f 0.58). IR (neat), cm^{-1}: 1725 (s), 1171, 1066; ^{1}H NMR, 300 MHz, $(CD_3)_2C{=}O$): δ 3.17 (m, 2H, benzyl methylene), 5.32 (m, 1H, $PhCH_2C\underline{H}$—), 5.47 (m, 1H, vinyl), 6.12 (m, 1H, vinyl), 6.33 (dd, 1H, $CH_2{=}C\underline{H}$ –, J = 1.2, 4.7, and 17.3 Hz), 7.24 (m, 5H, phenyl); ^{13}C NMR, 75 MHz, $(CD_3)_2C{=}O$): δ 37.05 (benzylmethylene), 72.85 (methyne —O—), 126.83 ($CH_2{=}CH$—), 128.01, 128.38, 129.45 and 136.78 (P<u>h</u>—), 131.22 ($\underline{C}H_2{-}CH$ –), 164.88 (—CO_2—), 169.97 (—COOH); CIMS: m/z 221 (M+H, 25%).

***N*-(Propiolyl)-L-phenylalanine (7).** *N*-Methylmorpholine (218 μL, 2.0 mmol) was added to a solution of L-phenylalanine methyl ester HCl (430 mg, 2.0 mmol) in 8 mL of a 1:1 mixture of anhydrous THF:DMF under an atmosphere of argon. The mixture was stirred for 5 min, followed by the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (382 mg, 2.0 mmol) and a dropwise addition of propiolic acid (123 μL, 2.0 mmol). The mixture was stirred in the dark for 16 h, and was then concentrated in vacuo to dryness. The residue was taken up in ethyl acetate, washed with 1 N HCl and water, and the organic layer was dried over anhyd Na_2SO_4. The organic solution was concentrated, and the compound was purified by flash chromatography on silica gel (30% ethyl acetate in hexane, R_f 0.15) to provide 200 mg of methyl *N*-(propioyl)-L-phenylalanine (48% yield). IR (neat), cm^{-1}: 3267, 2104, 1738, 1647; FABMS$^+$: 232 (M+H, 25%), 340 [M+H+108 (thiol matrix), 25%].

A suspension of methyl *N*-(propiolyl)-L-phenylalanine (220 mg, 0.95 mmol) in 10 mL of water was allowed to react with 50 mg of chymotrypsin (EC 3.4.21.1), which was dissolved in 5 mL of water. A constant pH of 7.2 was maintained by titrating the mixture with 50 mM NaOH using a pH-stat apparatus. The aqueous solution was washed with ether after 16 h, and was then acidified to pH 2.0 with 1 N HCl. The solution was concentrated to dryness in vacuo. The residue was sonicated in the presence of 50 mL ethyl acetate, and the resulting solution was filtered and dried over anhyd Na_2SO_4. Evaporation of the solvent provided 180 mg of the title compound (87% yield), which was found to be pure by TLC (85:10:5, chloroform:methanol:acetic acid, R_f 0.62). IR (neat), cm^{-1} 3271, 3032, 2114, 1730, 1643; ^{1}H NMR, 500 MHz, CD_3OD: δ 2.95 (dd, 1H, benzyl methylene, J = 9.4 and 14.0 Hz), 3.22 (dd, 1H, benzyl methylene, J = 4.9 and 14.0 Hz), 3.30 (m, 1 H, α-methine), 3.55 (s, 1H, acetylenic methyne), 7.24 (m, 6H, phenyl and NH); ^{13}C NMR, 125 MHz, CD_3OD: δ 36.67 ($PhC\underline{H}_2$—), 54.00 (—$\underline{C}H$—NH(CH_2Ph)–), 74.94 ($CH{\equiv}\underline{C}$ –), 76.40 ($\underline{C}H{\equiv}C$—) 126.46, 128.07, 128.76, 136.85 (—Ph–), 153.06 (—$\underline{C}$ONH—), 172.43 (—$\underline{C}O_2H$); EIMS m/z exp. 217.0740 (calcd 217.0738).

References and Notes

1. Ghosh, S. S.; Wu, Y. Q.; Mobashery, S. *J. Biol. Chem.* **1991**, *266*, 8759.

2. Wu, Y. Q.; Mobashery, S. *J. Med. Chem.* **1991**, *34*, 1914.

3. Ghosh, S. S.; Said-Nejad, O.; Roestamadji, J.; Mobashery, S. *J. Med. Chem.* **1992**, *35*, 4175.

4. Levy, O. E.; Taibi, P.; Mobashery, S.; Ghosh, S. S. *J. Med. Chem.* **1993**, *36*, 2408.

5. Ghosh, S. S.; Said-Nejad, O.; Mobashery, S. In *Peptides: Chemistry, Structure and Biology*; Hodges, R. S.; Smith, J. A., Eds.; Leiden, 1994; p 607.

6. (a) Suh, J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1976**, *98*, 1940; (b) Hilvert, D.; Gardell, S. J.; Rutter, W. J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1986**, *108*, 5298.

7. McClure, W. O.; Neurath, H. *Biochemistry* **1966**, *5*, 1425; Osumi, A.; Rahmo, A.; King, S. W.; Przystas, T. J.; Fife, T. H. *Biochemistry* **1994**, *33*, 14750.

8. Auld, D. S.; Vallee, B. L. *Biochemistry* **1970**, *9*, 602; Izumiya, N.; Uchio, H. *J. Biochem.* **1959**, *46*, 235.

9. For general discussion of the mechanism see: Matthews, B. W. *Acc. Chem. Res.* **1988**, *21*, 333; Christianson, D. W.; Lipscomb, W. N. *Acc. Chem. Res.* **1989**, *22*, 62, and ref cited therein.

10. Studies of mutant CPA variants with substitutions of Arg-127 indicated that the guanidinium moiety of arginine stabilizes the transition state of the rate-limiting step in the hydrolytic reaction by as much as 6 kcal mol^{-1}. Stabilization of the transition state was attributed to the electrostatic interactions of the guanidinium moiety with the oxyanion species (Phillips, M. A.; Fletterick, R.; Rutter, W. J. *J. Biol. Chem.* **1990**, *265*, 20692; Phillips, M. A.; Kaplan, A. P.; Rutter, W. J.; Bartlett, P. A. *Biochemistry* **1992**, *31*, 959). The potential polarization of the substrate carbonyl by Arg-127, as originally suggested by Lipscomb and colleagues, would facilitate the attack of the zinc-bound hydroxide ion at the carbonyl carbon, giving rise to the oxyanion in the transition state, which would be stabilized by the side chain of Arg-127.

11. Gilbert, J. B.; Price, V. E.; Greenstein, J. P. *J. Biol. Chem.* **1949**, *180*, 473; Putnam, F. W.; Neurath, H. *J. Biol. Chem.* **1946**, *166*, 603; Snoke, J. E.; Neurath, H. *J. Biol. Chem.* **1949**, *181*, 789; Neurath, H.; Schwert, G. W. *Chem. Rev.* **1950**, *36*, 69.

12. Kim, H.; Lipscomb, W. N. *Biochemistry* **1990**, *29*, 5546.

13. The binding mode of the small substrate Gly-Tyr to the enzyme is different from that for compounds **2**, **6**, and **7** since the active-site zinc is chelated by the amide carbonyl and the terminal free amine in this case (Christianson, D. W.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7568).

14. Gardell, S. J.; Craik, C. S.; Hilvert, D.; Urdea, M. S.; Rutter, W. J. *Nature (London)* **1985**, *317*, 551.

15. Suh and Kaiser suggested the possibility that the CPA-mediated hydrolysis of (+)-*S*-(*trans*-cinnamoyl)-α-mercapto-β-phenylpropionate may proceed via a transient

acyl-enzyme intermediate with a likely involvement of Tyr-248 (Suh, J.; Kaiser, E. T. *Biochem. Biophys. Res. Comm.* **1975**, *64*, 863). This initial observation was not followed up in the literature.

16. Hass, G. M.; Neurath, H. *Biochemistry* **1971**, *10*, 3535; Hass, G. M.; Neurath, H. *Biochemistry* **1971**, *10*, 3541.

17. Yun, M.; Park, C.; Kim, S.; Nam, D.; Kim, S. C.; Kim, D. H. *J. Am. Chem. Soc.* **1992**, *114*, 2281.

18. Edwards, B.; Tanaka, Y.; Mobashery, S. Unpublished results.

19. Simpson, R. T.; Riordan, J. F.; Vallee, B. L. *Biochemistry* **1963**, *2*, 616.

20. Gasteiger, J.; Marsili, M. *Tetrahedron* **1980**, *36*, 3219; Gasteiger, J.; Marsili, M. *Org. Magn. Reson.* **1981**, *15*, 353

21. Stein, W. H.; Moore, S. *J. Biol. Chem.* **1948**, *176*, 367.

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