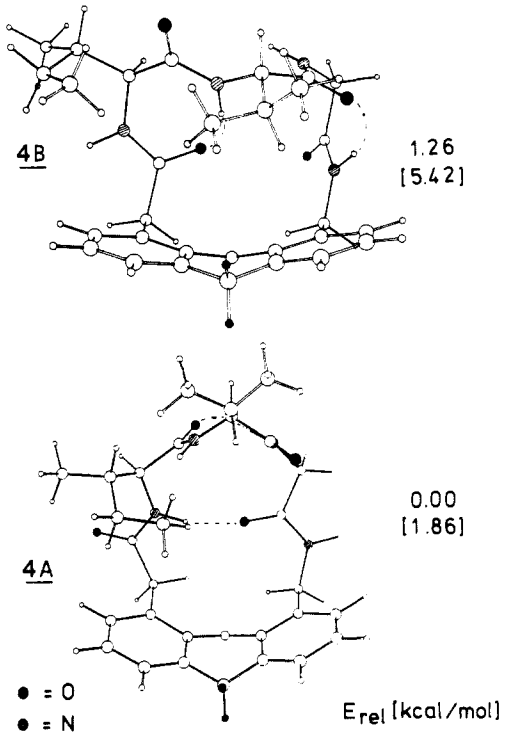

Figure 1. Geometries and relative steric energies of the conformations **4A,B** derived from force-field calculations. The numbers in brackets refer to alternative conformations (not shown) in which the phenoxathiin part is bent away from the view point. Aromatic methyl groups are replaced by hydrogens.

Table I. Comparison of Proton Distances r_{ij} Derived from NOE Experiments with the Minimum-Energy Geometries **4A,B**

connected protons	exptl r_{ij}[a]		force field	
	2D NMR	1D NMR	**4A**	**4B**
I, Ile-NH-**1**-CH_2CO[b]	2.44	2.24	2.43	2.53
II, Val-NH-Ile-βH	2.35	2.26	2.36	4.52
III, Gly-NH-Val-αH	2.37	2.30	2.43	2.92
IV, **1**-NH-**1**-CH_2N[c]	2.32	2.25	2.26	2.64
V, **1**-CH_2CO-**1**-CH_2N[d]	2.52	*e*	2.26	2.93

[a] Values in Å, error limits ca ±0.2. [b] *Pro-S* proton in **4A**; *Pro-R* proton in **4B**. [c] *Pro-S* proton. [d] *Pro-R* proton. [e] A selective irradiation in the 1D experiment is impossible due to the small chemical shift difference.

A representative 2D NOE spectrum is shown in Figure 2.

Distances between protons were calculated in two ways—from the buildup rate of the 2D NMR cross-peak intensities with increasing mixing time[9] and from time-dependent 1D NOE experiments.[11,12] The results of both techniques are compared with the minimum-energy geometries in Table I. The experimental evidence suggests that **4A** is the observed low-energy conformation. **4B** can be eliminated for it has two r_{ij} which are to long to explain the NOE buildup rates. The γ-loop involving the Val residue in **4A** is in concordance with the II and III NOE effects (Table I). However, the temperature coefficient of the presumably hydrogen-bonded Gly-NH (see above) indicates exposure to the solvent; so the local conformation at the Val residue may be still flexible.

In summary, three independent NMR experiments—NH temperature shifts, coupling constants, and NOE connectivities—support structure **4A** which contains the anticipated

(9) (a) Kumar, A.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *J. Am. Chem. Soc.* **1981**, *103*, 3654-3658. (b) Bruch, M. D.; Noggle, H. J.; Gierasch, L. M. *J. Am. Chem. Soc.* **1985**, *107*, 1400-1407.

(10) Macura, S.; Huang, Y.; Suter, D.; Ernst, R. R. *J. Magn. Reson.* **1981**, *43*, 259-281.

(11) Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 1332-1339 and references cited therein.

(12) Known distances were used for calibration, e.g., the geminal protons of **1** or of Gly in **4**.

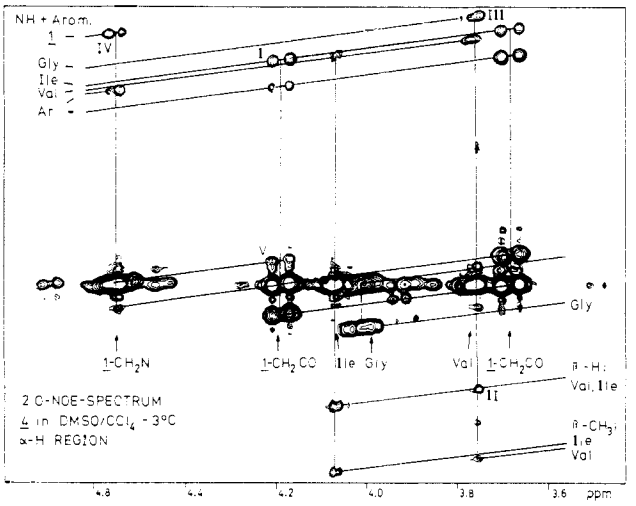

Figure 2. αH region of the 400-MHz 2D NOE spectrum of **4** in Me_2SO-d_6/CCl_4 at −3 °C. The echo pulse sequence (90°-t_1/2-90°-t_m-90°-t_1/2-FID) was used. A small random variation (±5 ms) of the mixing time t_m (375 ms) was applied to cancel unwanted signals due to J coupling.[10] The spectrum is recorded in the N-type mode which gives the somewhat unusual direction of the lines of connectivity. The numbers I to V refer to Table I.

hydrogen bridge of a β-loop type. The structure resembles cyclic pentapeptides—compounds that contain normally at least one D-amino acid or glycine. Similar cycles with different amino acid compositions are being synthesized in order to show whether the rules derived for the conformation of cyclic pentapeptides[13] are still applicable when **1** substitutes two of the amino acids.

Acknowledgment. This work was supported by the Deutsche Forschungsgemeinschaft.

(13) Kessler, H.; Hehlein, W.; Schuck, R. *J. Am. Chem. Soc.* **1982**, *104*, 4534-4540. (b) Gierasch, L. M.; Karle, I. L.; Rockwell, A. L.; Yenal, K. *J. Am. Chem. Soc.* **1985**, *107*, 3321-3327.

Productive Conformation in the Bound State and Hydrolytic Behavior of Thiopeptide Analogues of Angiotensin-Converting Enzyme Substrates

Louise Maziak, Gilles Lajoie, and Bernard Belleau*

Department of Chemistry, McGill University
Montreal (Quebec), Canada H3A 2K6

Received August 23, 1985

We wish to report the unexpected behavior of angiotensin-converting enzyme (ACE; dipeptidyl carboxypeptidase EC 3.4.15.1) toward the thiopeptide analogues *N*-(furylacryloyl)-L-thiophenylalanylglycyl-L-proline [FA-Phe-Ψ-(CSNH)-Gly-Pro, **1**] and *N*-(furylacryloyl)-L-thiophenylalanyl-L-alanyl-L-proline [FA-Phe-Ψ-(CSNH)-Ala-Pro, **3**][1] of the well-known tripeptide substrates **2** and **4** (Table I). These thioamide analogues appeared attractive as potential ligands of the active-site zinc ion of the enzyme, the thiocarbonyl function being susceptible in principle to effective coordination by the metal. We found that thiopeptide **1** suffers ready hydrolysis by ACE at a rate comparable to that of **2** whereas analogue **3**, in contrast to the choice parent substrate **4**, is not hydrolyzed even over extended periods of time. This good substrate property of **1** was unexpected on the basis of the reported behavior of thioamide analogues of peptide substrates toward carboxypeptidase A (CPA),[3,4] an enzyme whose catalytic mech-

(1) For IUPAC-IUB nomenclature, see: *Eur. J. Biochem.* **1984**, *138*, 9.

(2) Holmquist, B.; Bunning, P.; Riordan, J. F. *Anal. Biochem.* **1979**, *95*, 540.

(3) Campbell, P.; Nashed, N. T. *J. Am. Chem. Soc.* **1982**, *104*, 5221.

0002-7863/86/1508-0182$01.50/0

Table I. Summary of Kinetic Parameters for ACE Substrate Analogues

	K_m, M	k_{cat}, s^{-1}
Fa-Phe-Ψ-(CSNH)-Gly-Pro (**1**)	1.4×10^{-3}	16000
FA-Phe-Gly-Pro (**2**)	4.4×10^{-3}	41000
FA-Phe-Ψ-(CSNH)-Ala-Pro (**3**)	*a*	*a*
FA-Phe-Ala-Pro (**4**)	3.8×10^{-4}	11000

[a] Not hydrolyzed in extended assay periods (16 h).

Table II. ^1H NMR Parameters for ACE Substrate Analogues

	ppm X 10^{-3}/°C			$^3J_{\alpha\beta}$ of
	NH-Phe	NH-Ala	NH-Gly	Phe/Hz
Bz-Phe-Ψ-(CSNH)-Gly-Pro (**5**)	4.8		4.3	
Bz-Phe-Gly-Pro (**6**)	5.2		5.7	
Bz-Phe-Ψ-(CSNH)-Ala-Pro (**7**)	5.2	5.7		4.3, 10.8
Bz-Phe-Ala-Pro (**8**)	5.0	5.9		4.4, 10.6

anism is believed to parallel that of other zinc-dependent enzymes such as thermolysin and ACE.[5] In the case of CPA, Campbell and Nashed[3] and Bartlett et al.[4] have demonstrated that although thionation of the scissile amide bond of a peptide substrate minimally affected the affinity for the active site, it markedly hindered the catalytic process. Our findings thus unravel unsuspected stereoelectronic features of the ACE active site when viewed in the light of our current understanding of the mechanism of zinc-dependent peptidases.[2,3,5]

Analogues **1** and **3** were selected for our studies because the parent peptides are known to be well accepted as substrates by ACE.[2] On the basis of a hypothetical active-site model for ACE,[6] one may expect the thioamide sulfur of analogues **1** and **3** to interact readily with the zinc ion either in a catalytically productive or unproductive manner. Thiopeptides **1** and **3** were prepared by the selective thionation of the methyl esters of the *N*-Boc-protected tripeptide precursors using a soluble thionating agent under mild conditions as previously reported.[7] The *N*-furylacryloyl derivatives were then generated from the deprotected intermediates in the conventional manner and their behavior toward ACE was monitored according to the method of Bunning et al.[8] All substrate analogues were dissolved in phosphate buffer (pH 7.8) at 25 °C followed by the addition of enzyme. The mixtures were monitored for hydrolysis at λ = 345 nm for **1** and 340 nm for **3** using a Varian Cary 210 spectrophotometer. Toward ACE, **1** exhibited conventional Michaelis–Menten behavior under conditions where [S] >> $[E]_0$; the kinetic parameters K_m and k_{cat} were 1.4 mM and 1.6×10^4 s^{-1}, respectively. Under identical conditions, **3** was not hydrolyzed even after a 16-h incubation period. For practical purposes, the *N*-benzoyl analogue **7** of **3** was also prepared by the same methods and found to behave as a pure competitive inhibitor of the substrate FA-Phe-Gly-Gly,[8] with K_i = 4 mM. The conclusion is inescapable then that it is the combined presence in **3** of the alanyl side chain methyl group and the sulfur atom of the thioamide function that confers complete resistance to hydrolysis and that decreases the affinity of the tripeptide for the ACE binding site.

The question of the possible effect(s) of the thioamide function on the solution conformation of relevant parent peptides was examined in a preliminary fashion by ^1H NMR spectroscopy. The possibility of sluggish cis–trans isomer interconvertibility about

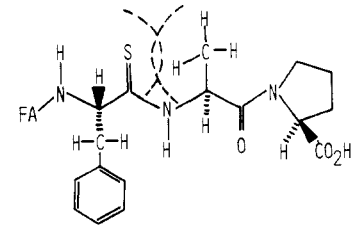

Figure 1. Schematic representation of the productive conformation of ACE-bound substrates which is disfavored by the simultaneous presence of a side chain methyl and a thiocarbonyl group at the scissile bond.

the thioamide function is of some concern because of the demonstration that certain proteases catalyze hydrolysis of trans peptide bonds exclusively.[9,10] However, we observed a splitting of the resonance signal for the alanyl amide proton of **7** reflecting the presence of cis–trans isomers in a ratio of 5:95. In addition, the hydrogen-bonding properties of **5** and **7** were contrasted with those of **6** and **8**, respectively, by temperature-dependence studies in Me_2SO-d_6. As shown in Table II, all compounds showed similarly large temperature coefficients, indicating that none engage in intramolecular hydrogen bonding nor do the pairs **5** and **6** or **7** and **8** experience different shielding environments. Moreover, the vicinal coupling constants $^3J_{\alpha\beta}$ of the phenylalanyl residues in **7** and **8** were of similar magnitude (Table II), thereby indicating that the χ_1 angle of Phe is similar in both compounds.[11] Accordingly, the presence of a thiopeptide linkage does not appear to significantly alter the ground-state solution conformation of the parent peptide in Me_2SO-d_6.

Although it has been postulated that effective coordination of a peptide substrate with the CPA active site would require twisting of the scissile bond[12–14] and that thioamides are known to display a higher barrier to rotation than amides,[15] the contrasting behavior of **1** and **3** toward ACE requires an explanation based on an enzyme-imposed productive conformation easily attained by **1**, **2**, and **4** but not by **3**. Thus, whereas a frozen conformation requiring the oxygen of the scissile bond to lie in or near the plane of the alanyl methyl group poses no difficulty for **4**, the same conformation is markedly disfavored in **3** because the increased length of the thiocarbonyl bond together with the larger size of the sulfur atom precludes the side chain methyl from lying in the plane of the sulfur atom.[16] This disfavored conformation of **3**, which is shown in Figure 1, neatly accommodates the substrate properties of analogue **1**, a fact of mechanistic significance for zinc-dependent peptidases.[17,18] These novel stereoelectronic features of the ACE active-site selectivity and reactivity offer interesting opportunities for mechanistic studies and the design of new types of potentially useful inhibitors.

Acknowledgment. We thank the N.S.E.R.C. Canada for financial support and Dr. Françoise Sauriol for the ^1H NMR results and Antonia DiPaola for skillful assistance with the enzyme preparation. We are grateful to Dr. Andrê Michel, University of Sherbrooke, for the hard-sphere calculations.

Supplementary Material Available: Infrared, proton magnetic resonance, mass spectroscopic, and/or microanalytical data are available (1 page). Ordering information is given on any current masthead page.

(4) Bartlett, P. A.; Spear, K. L.; Jacobsen, N. E. *Biochemistry* **1982**, *21*, 1608.
(5) Hangauer, D. G.; Monzingo, A. F.; Matthews, B. M. *Biochemistry* **1984**, *23*, 5730. Monzingo, A. F.; Matthews, B. M. *Biochemistry* **1984**, *23*, 5724.
(6) Petrillo, E. W.; Ondetti, M. A. *Med. Res. Rev.* **1982**, *1*, 1.
(7) Lajoie, G.; Lêpine, F.; Maziak, L.; Belleau, B. *Tetrahedron Lett.* **1983**, 3815.
(8) Bunning, P.; Holmquist, B.; Riordan, J. F. *Biochemistry* **1983**, *22*, 103.
(9) Lin. L. N.; Brandts, J. F. *Biochemistry* **1979**, *18*, 43.
(10) Lin, L. N.; Brandts, J. F. *Biochemistry* **1979**, *18*, 5037.
(11) Feeny, J. *J. Magn. Reson.* **1976**, *21*, 453.
(12) Mock, W. L. *Bioorg. Chem.* **1975**, *4*, 270.
(13) Cleland, W. W. *Adv. Enzymol. Mol. Biol.* **1977**, *45*, 273.
(14) Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3875.
(15) Leopardi, C. P.; Fabre, O.; Zimmeman, D.; Russi, J.; Cornea, F.; Fulea, C. *Can. J. Chem.* **1977**, *55*, 2649.
(16) Hard-sphere calculations using computer-assisted molecular modeling showed passing interaction energies of 1, 1, 28.8, and 9.3 kcal/mol for compounds **1**, **2**, **3**, and **4** (Table I), respectively, thus supporting our interpretation.
(17) Rees, D. C.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 7151.
(18) Auld, D. S.; Galdes, A.; Geoghegan, K. F.; Holmquist, B.; Martinelli, R. A.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 5041.